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Indications	T Cells				Peptides									
	T _{MBP}	T _{MBPC}	T _{NOGA}	T _{Cop1}	MBP	MBP _c	MBP _o	MOG	PLP enc	PLP nenc	Noga	IRBP	Cop1	Other
optic crush	ES2 ES2B 2,8' 10,27	ES2 ES2B 7	ES13B ES18		ES2 ES2B 41	ES2 ES2B 12	12	12	32	7	ES13B ES18			
spinal cord	ES2 ES2B 5,9 14,20		ES31								ES2B		ES31 (sph, G91,A91 A96)	
IOP			23	9 20							40	ES13B ES18	40	
facial nerve											37			
closed head											39			
glutamate			ES13B ES18								ES13B ES18 15			
motor neuron diseases											ES35			
Huntington's disease											ES32			



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2: MOALEM et al, *Nature Medicine*, 5:49-55 (1999)
5: HAUBEN et al, *The Lancet*, 354:286-287 (2000)
7: KIPNIS et al, *PNAS*, 97:7446-7451 (2000)
8: MOALEM et al, *J. Neuroimmunol.*, 106:189-197 (2000)
9: HAUBEN et al, *J. Neurosci.*, 20:6421-6430 (2000)
10: MOALEM et al, *J. Autoimmun.*, 15:331-345 (2000)
12: FISHER et al, *J. Neurosci.*, 21:136-142 (2001)
14: BUTOVSKY et al, *FASEB J.*, express article 10.1096/fj.00-0550fje (2001)
15: SCHORI et al, *PNAS*, 98:3398-3403
20: HAUBEN et al, *J. Clin. Invest.*, 108:591-599 (2001)
23: HAUBEN et al, *PNAS*, 98:15173-15178 (2001)
27: BAROUCH et al, *FASEB J.*, 16:1304-1306 (2002)
32: MIZRAHI et al, *Euro. J. Neurosci.*, 16:1731-1740 (2002)
37: ANGELOV et al, *PNAS*, 100:4790-4795 (2003)
39: KIPNIS et al, *J. Neurotrauma*, 20:559-569 (2003)
40: BAKALASH et al, *Invest. Ophthalmol. Vis. Sci.*, 44:3374-3381 (2003)
41: MONSONEGO et al, *J. Autoimmun.*, 21:131-138 (2003)





A common vaccine for fighting off neurodegenerative disorders: Recharging immunity for homeostasis

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Teaser: In neurodegenerative diseases there is an incompatibility between the need for assistance from the immune system and its ability to supply it; this ties up with the paradoxical nature of remedial autoimmunity — that the cells which protect against neurodegeneration may be the very cells that mediate development of an autoimmune disease.

Abstract

Neurodegenerative conditions share some common primary risk factors and mediators of disease progression. Since many degenerative disorders are age-related, deteriorating immunity in aging patients might impose additional risk. Adaptive (T cell-mediated) immunity is a defense mechanism instructing microglia to fight off and clear away self-derived enemies. It can be boosted, without risking autoimmune disease, by injecting weak agonists of self-antigens or by weakening the suppressive CD4⁺CD25⁺ regulatory T cells. If widely cross-reactive, the same agonist might effectively counteract a variety of neurodegenerative disorders. Boosting of relevant T cells can thus “recharge” a deteriorating immune system that has to contend with increasing risk factors.

Common features of neurodegenerative disorders

Neurodegenerative diseases are generally considered to be noninflammatory, unlike autoimmune diseases such as multiple sclerosis, which are both inflammation-related and neurodegenerative [1,2]. It is becoming increasingly clear that degenerative diseases of the central nervous system (CNS) share several primary and secondary features [3-6]. Primary risk factors include self-compounds that accumulate extracellularly in toxic amounts or in nonphysiological conformations [7]. The self-perpetuating spread of damage that follows acute injury, or occurs independently of primary risk factors in any chronic neurodegenerative disorder, is commonly viewed as secondary degeneration [8]. The mechanisms that underlie the secondary degeneration include biochemical and metabolic changes in oxygen and glucose utilization, in energy state, and in lipid-dependent enzymes, free radicals, eicosanoids, tissue ions, biogenic amines,

endogenous opioids, and excitatory amino acids. These changes cause alterations in cellular homeostasis, excitotoxicity, local production of agents harmful to nerve cells, and loss of trophic support from targets, all resulting in neuronal loss (Figure 1). In addition, many neurodegenerative diseases are characterized by the presence of activated microglia, which, owing to the phenotype they acquire, are well equipped with defense mechanisms for killing microorganisms but are poorly tolerated by the brain. Thus, not only do they not contribute to repair but they play a key role in the ongoing pathology [6]. Such activated microglia serve as a source of inflammatory compounds such as tumor necrosis factor (TNF)- α and cyclooxygenase-2, whose cytotoxic activity contributes to the ongoing degeneration [9,10].

Intensive research has therefore been devoted to finding compounds capable of counteracting the mediators of toxicity or deactivating the microglia. An inherent limitation of such approaches is that many of the mediators are compounds, which at physiological concentrations are essential for the well-being of the brain; neutralizing them might therefore simply replace the harmful excess with harmful deficiency. Likewise, as discussed below, suppression of microglia would inevitably exclude any beneficial activity that might be gained by activating them in a different way. Studies have indeed shown that unless special weak antagonists (e.g. memantine) are employed [11], the use of pharmacological agents to block brain neurotransmitters inevitably causes adverse side effects owing to the pivotal physiological role of these neurotransmitters in brain function. As an example, treatment of stroke victims with glutamate-receptor antagonists carries the risk of inducing hypoglutamatergic conditions, with devastating consequences for neuronal survival and brain function [12]. Similarly, while brain-derived neurotrophic factor is essential for neuronal survival, overdosing causes neurotoxicity by evoking inducible nitric oxide synthase, with consequent increase in NO production [13]. Thus,

pharmacological intervention requiring fine-tuning of the molecular players in brain functioning and maintenance, many of which have context-dependent dual (and opposing) effects, carries some risks.

An additional obstacle to therapy is that in some of these diseases the degeneration continues even overcoming the primary cause. In Parkinson's disease, for example, despite replacement therapy with dopamine (L-dopa) the dopaminergic neurons continue to die [14]. Similarly, in Alzheimer's disease, the extracellular environment remains hostile to healthy neurons even after therapeutic removal of amyloid plaques [15]. In glaucoma, another neurodegenerative disease, reduction of intraocular pressure often does not stop disease progression [16,17].

Findings over the past few years have suggested that a more global therapy might be achieved by harnessing the body's own fighting force. Harnessing of the immune system, for example, might provide a comprehensive way to address the multifactorial nature of neurodegenerative diseases and the multiple actions of cells and compounds in the CNS. The background to such a global approach is summarized below.

Protective autoimmunity—from experimental paradigm to physiological response

Early studies by our group demonstrated, contrary to the prevailing dogma, that recovery of acutely injured CNS neurons requires the activity of immune cells directed against self-proteins residing in the site of damage [18]. This finding led to formulation of the novel concept of protective autoimmunity (see text box) [19]. This idea, although still regarded by some scientists as a scientific caprice, has sparked a spate of investigations of neuro-immune interactions, with highly promising results. Injection of autoimmune T cells reactive to myelin basic protein (MBP)

into rats after a severe traumatic injury to the optic nerve or spinal cord, provided that the accompanying experimental autoimmune encephalomyelitis (EAE) is mild, confers significant protection on neurons that escaped the primary injury, and thus dramatically increases neuronal survival [18,20]. Survival of neurons is accompanied by improved functional activity and is long-lasting [20]. Similar results, without the accompanying autoimmune disease, are obtained with anti-self T cells directed to a nonencephalitogenic epitope of MBP [18]. The T cells used in these early experiment were Th0 which, when activated, are capable of producing cytokines characteristic of both Th1 and Th2 ([21]. Passive transfer of Th1 clones into T cell-deficient mice causes EAE, but does not confer neuroprotection [22]. The beneficial effect of the accumulated autoimmune T cells, once locally activated, is expressed in modulation of the local glial response to injurious conditions [23,24]. Moreover, such T cells upon activation produce neurotrophic factors [25]. Other groups have also reported that absence of adaptive immunity exacerbates postinjury neuronal degeneration [26-28] and that autoimmune T cells, by producing neurotrophic factors, beneficially affect neural tissue [29,30].

Several methods were employed in our laboratory to demonstrate that autoimmunity is a physiological repair mechanism, operating with a rapid on/off switch to meet the requirement of maintaining homeostasis without risk of autoimmune disease [31]. Adult mice that were rendered incapable as neonates of responding to specific antigens residing in the site of damage were found to lose more neurons after a CNS injury than normal controls [32]. In addition, significantly more neurons survive CNS insults in mice depleted of naturally occurring CD4⁺CD25⁺ regulatory T cells (Treg) than in wild-type mice [32,33].

On the basis of the results a paradigm shift in the perception of autoimmune disease was proposed, in which autoimmune disease is viewed as the result of the body's failure to control the

genetically determined spontaneous protective autoimmune response to an injury. Moreover, the results led to recognition of the paradoxical nature of autoimmunity — or in other words, that the cells which protect against neurodegeneration are apparently the very cells that mediate development of an autoimmune disease in susceptible individuals [34]. Therefore, for remedial autoimmunity a fine balance must be achieved between boosting of the physiological level of autoimmunity and avoiding autoimmune diseases. In individuals resistant to autoimmune diseases the balance is not critical, as the spontaneous response is of low affinity and is therefore not likely to be encephalitogenic. To avoid exceeding the threshold the antigens used were altered peptide ligands, self-peptides in which certain amino acid alterations result in weak (and therefore nonencephalitogenic) agonist activity [35].

Immunity and autoimmunity in the CNS in health and disease: retrospective and prospective

Can the concept of protective autoimmunity be reconciled with the dogma of tolerance to self? Autoimmunity, particularly in the context of the CNS, has long puzzled scientists. Detection of autoimmune T cells in the brain was first reported in patients with multiple sclerosis [36,37]. Autoimmune T cells were viewed as thymic escapees, and a predisposition to development of organ-specific autoimmune diseases was assumed to result from the presence of autoimmune T cells in the periphery [38]. During the 1990s it was proposed that a newly identified subpopulation of naturally occurring T cells, the regulatory CD4⁺CD25⁺ T cells (Treg), serves primarily to keep autoimmune T cells in a state of tolerance (i.e., nonresponsiveness) in the periphery, i.e., to suppress thymic escapees [39]. In the mid-1990s, Sakaguchi and his colleagues showed that depletion of Treg speeds up tumor rejection in mice [40]. Treg depletion also predisposes the mice to spontaneous development of an autoimmune disease [41].

In light of the suppressive effect of Treg on the ability to cope with stressful conditions in the CNS [32,33], it is interesting to note that in both EAE-resistant and EAE-susceptible animal strains removal of Treg improves the ability to cope with the stress, but that whereas in the wild type of resistant strains exogenous Treg diminishes the spontaneous ability to withstand the degenerative conditions, in the wild type of susceptible strains exogenous Treg (administered on top of the existing Treg) improves such ability [33]. These findings point to a difference in the default equilibrium between autoimmune effector ($CD4^+CD25^-$) T cells and Treg in strains that are susceptible and resistant to development of EAE, and suggest, apparently paradoxically, that Treg activity is higher in susceptible than in resistant strains (Figure 2). They further suggest that Treg, as well as suppressing the protective activity of effector T cells, can themselves be protective if the usual protective T cells are absent. Passive transfer of Treg into immunodeficient mice of a genetically resistant background strain indeed had a protective effect. These finding serve as a warning that since immune-related therapies based on induction of regulatory T cells appear to be at least partially dependent on the inherent genetic endowment [42,43], they might not be universally beneficial.

To what extent is the concept of “protective immunity” corroborated by the work of other laboratories?

From the numerous recent publications addressing the role of inflammation in CNS tissue repair, particularly after spinal cord injury and in chronic neurodegenerative diseases, it has become clear that immunosuppression as a therapeutic approach is at best ineffectual and at worst destructive [44,45]. Moreover, a more precise definition is needed in order to characterize the classical inflammatory response and distinguish it from the local immune response. Thus, for

example, whereas the CNS is barely able to tolerate the former, it can derive substantial benefit from an adaptive immune response whose timing and intensity are well-controlled and in which both pro-inflammatory cytokines such as IFN- γ and anti-inflammatory cytokines such as IL-4 and IL-10 are produced. Each of these cytokines, when present at the right time and in well-controlled amounts, might be beneficial for the tissue, but if uncontrolled (e.g. excessive amounts of IFN- γ or inappropriately timed (e.g., IL-10 [46]) or present in an unfavorable combination [33] they might have adverse effects. This might explain some of the reported discrepancies in the findings of different laboratories in connection with the effects of autoimmunity obtained by passive or by active vaccination.

Other apparent discrepancies in the findings from different laboratories might arise from variations among animals from center to center, or from other conditions that can lead to diseases of different severities even if ostensibly the same immunization protocols are used (see, e.g., [47]). In such cases, inexperienced authors might fail to recognize that their results, far from contradicting the concept of protective immunity, actually confirm and strengthen it. Thus, for example, active immunization of CNS-injured rodents with MPB in amounts that exceed the critical dose threshold for the induction of clinical and pathological features of EAE [48,49] evokes an autoimmune response which will almost certainly lead to disease of such severity that it outweighs the protective effect; as a result, the overall outcome will not be better and might even be worse than in nonimmunized controls [50].

David and his colleagues showed that immunization with myelin or with non-encephalitogenic myelin peptides (using a protocol that did not result in EAE development in any of the immunized mice) induces neuroregeneration [51,52]. The group of Olsson and Piehl [29,53] showed that in mice devoid of T cells neuronal survival after injury is decreased, and that

autoimmune T cell produce neurotrophins that might benefit injured neurons. Hofstetter et al. [54] showed that autoimmunity to myelin oligodendrocyte protein (MOG) in C57.BL/6 mice with aseptic cerebral injury accelerates both revascularization and post-traumatic healing. Rutkowski et al. [55] suggested a role for protective CNS autoimmunity following peripheral nerve injury. Schwab and colleagues recently described their use of a nonencephalitogenic immunization protocol with MOG or Nogo-A [56,57].

Therapeutic vaccines

Boosting of autoimmunity as a possible remedy for neurodegenerative diseases is reminiscent of the use of attenuated or weakened pathogens in a prophylactic vaccination designed to educate the immune system to withstand an aggressive microbial invasion. The possibility of using altered self-antigens (weak agonists of dominant antigens) as a protective vaccine against degenerative conditions in laboratory animals was explored by our group [21,50]. Immunization with self-peptides significantly improves neuronal survival [22]. Such peptides should be derived from site specific immunogenic self-proteins regardless of the type of lesion [58].

A weak agonist capable of activating a wide range of autoreactive T cells can potentially provide a single therapy for various neurodegenerative conditions in various sites. One such compound is glatiramer acetate, also known as copolymer-1 (Cop-1, Copaxone®), an approved drug for treatment of patients with multiple sclerosis [59]. This copolymer can itself activate a wide range of autoimmune T cells [60].

A single immunization with Cop-1 was found to evoke a T cell response. Such T cells are attracted by certain self-antigens residing in damaged CNS sites, where they accumulate and become activated that results in production of cytokines and growth factors, but not in T cell proliferation. Since Cop-1 can overcome the tissue-specificity barrier [61], its influence on neuronal survival in animal models of CNS degeneration is striking [21,61]. It exerts its effect in models of white matter (axonal) injuries, which respond to immunization with MBP, as well as in models of gray matter damage, where MBP has no effect and only site-specific dominant proteins are neuroprotective [61,62]. In addition, Cop-1-reactive T cells were neuroprotective not only after acute injury but also in models of chronic neurodegeneration, e.g. glaucoma [63], amyotrophic lateral sclerosis (ALS) [62], Parkinson's [64], Alzheimer's (unpublished data), and other diseases [65]. It thus seems, because of the wide-ranging cross-reactivity and the common therapeutic target (microglia) within the diseased tissue, that the same vaccination is likely to be effective in treating a wide range of neurodegenerative diseases.

Immune-based therapy for noninflammatory neurodegenerative diseases was recently attempted in animal models of Alzheimer's and prion diseases. In both cases it was suggested that plaque might be cleared, at least in part, by antibodies directed against components associated with its formation [66,67]. Whether antibody production, like autoimmune T cell activity, also serves as a physiological defense mechanism against such self-destructive compounds has never been investigated. Moreover, patients with Alzheimer's disease demonstrate large numbers of autoimmune T cells directed against β -amyloid, a principal component of plaque [68]. Preliminary results suggest, however, that the target of protective therapy mediated by autoimmune T cells cannot be the threatening compound itself (e.g., β -amyloid), but dominant self-peptides that are part of the protein component that normally resides

in the site of damage (unpublished observations). Thus, the presence of autoimmune T cells specific to β -amyloid might be of diagnostic significance, but it is not a therapeutic tool for either boosting or suppression. This of course does not exclude the use of antibody as a possible therapeutic target.

Vaccination is not the only possible immune-based neuroprotective therapy. Any immune manipulation that activates the immune system in a way that promotes the arrival of autoimmune T cells at the injured site is likely to be beneficial. Two novel ways of obtaining the desired effect were recently discovered: induction of lymphopenia [69] or weakening of naturally occurring Treg [32].

Induction of lymphopenia significantly increases immunoreactivity towards cancer-specific proteins and efficiently suppresses cancer [70]. A sudden drop in the pool of peripheral T lymphocytes stimulates their homeostasis-driven proliferation in order to restore the pool. In response to the stimulus of lymphopenia, naïve peripheral T cells proliferate and acquire a phenotype reminiscent of memory T cells [71]. Under lymphopenic conditions T cells can proliferate upon interaction with MHC-II molecules alone, with no need for a co-stimulatory signal [72]. If at the time of lymphopenia induction the body is undergoing stress, with consequent exposure of certain self-antigens (related, for example, to tissue injury or cancer), it will produce a T cell response to those antigens. Lymphocytes produced as a result of the irradiation-induced recovery of homeostasis will include a relatively high incidence of specific autoimmune T cells [73]. In rodents suffering from acute or chronic neurodegenerative conditions, induction of lymphopenia is of significant benefit for postinjury neuronal survival [33]. Lymphopenia and the subsequent homeostatic proliferation can be induced in several ways, the most clinically relevant being low-dose irradiation of the lymphoid organs. Owing to the

lymphopenia, T cells proliferate and become activated. These cells patrol the body, and since (being activated) they can cross the blood–brain barrier, their patrol route includes the CNS. On reaching the lesion site, and after becoming activated there by resident cells that present self-antigens in the MHC-II groove, T cells perform their effector functions, similarly to Cop-1-reactive T cells or T cells obtained by immunization with self- or altered self-proteins [33].

Concluding remarks

Harnessing the immune system in a well-controlled way might be the therapy of choice for neurodegenerative disorders (Figure 1). It is becoming clear that the T cell homeostasis of the brain is not restricted to motor performance controlled by the brain, but also includes higher brain functions. Thus, as long as the integrity of the immune system is maintained and neurotransmitter imbalance in the brain is still within the remediable capacity of the immune system, homeostasis remains intact and the integrity of brain performance is preserved. According to this view, therefore, the widening age-related gap between deteriorating immunity and risk factors for diseases can be narrowed by appropriate activation of the immune system.

Text Boxes

Commonality of acute and chronic CNS neurodegeneration – The self-perpetuating spread of degenerative damage that follows an acute injury, or occurs independently of the nature of the primary risk factors in any chronic neurodegenerative disorder, is termed secondary degeneration. Both acute and chronic CNS neurodegeneration are amenable to neuroprotective therapy, which prevents or reduces secondary degeneration and rescues marginally damaged neurons.

Protective autoimmunity – The body's fighting force against the enemy within (i.e., against destructive self-compounds). The antigenic specificity of the anti-self T cells that mediate a protective autoimmune response is determined by antigens that are abundantly expressed at the damaged site. This T cell-mediated autoimmune response is protective as long as its intensity and timing are well regulated. If not properly controlled the response can turn destructive, inducing a tissue-specific autoimmune disease.

Autoimmune disease – An outcome of the failure to properly control autoimmunity.

Tolerance to self-proteins – Formerly defined as ‘nonresponsiveness to self’; currently viewed as the ability to tolerate a response to self-proteins without developing an autoimmune disease.

References

- 1 Hohlfeld, R. and Wekerle, H. (2001) Immunological update on multiple sclerosis. *Curr. Opin. Neurol.* 14, 299-304
- 2 Silverstein, A.M. and Rose, N.R. (1997) On the mystique of the immunological self. *Immunol. Rev.* 159, 197-206; discussion 207-118
- 3 Brown, G.C. and Bal-Price, A. (2003) Inflammatory neurodegeneration mediated by nitric oxide, glutamate, and mitochondria. *Mol. Neurobiol.* 27, 325-355
- 4 Vila, M. and Przedborski, S. (2003) Targeting programmed cell death in neurodegenerative diseases. *Nat. Rev. Neurosci.* 4, 365-375
- 5 Orrenius, S. et al. (2003) Regulation of cell death: the calcium-apoptosis link. *Nat. Rev. Mol. Cell. Biol.* 4, 552-565
- 6 Jellinger, K.A. (2003) General aspects of neurodegeneration. *J. Neural Transm. Suppl.*, 101-144
- 7 Mattson, M.P. (2002) Oxidative stress, perturbed calcium homeostasis, and immune dysfunction in Alzheimer's disease. *J. Neurovirol.* 8, 539-550
- 8 Harrop, J.S. et al. (2001) The cause of neurologic deterioration after acute cervical spinal cord injury. *Spine* 26, 340-346
- 9 Monsonego, A. et al. (2003) Microglia-mediated nitric oxide cytotoxicity of T cells following amyloid beta-peptide presentation to Th1 cells. *J. Immunol.* 171, 2216-2224
- 10 Tanaka, M. et al. (1994) Detection of superoxide production by activated microglia using a sensitive and specific chemiluminescence assay and microglia-mediated PC12h cell death. *J. Neurochem.* 63, 266-270

- 11 Smith, P.F. (2003) Therapeutic N-methyl-D-aspartate receptor antagonists: will reality meet expectation? *Curr. Opin. Investig. Drugs* 4, 826-832
- 12 Muir, K.W. and Lees, K.R. (1995) Clinical experience with excitatory amino acid antagonist drugs. *Stroke* 26, 503-513
- 13 Takumida, M. and Anniko, M. (2002) Brain-derived neurotrophic factor and nitric oxide synthase inhibitor protect the vestibular organ against gentamicin ototoxicity. *Acta Otolaryngol.* 122, 10-15
- 14 Hirsch, E.C. (1999) Mechanism and consequences of nerve cell death in Parkinson's disease. *J. Neural Transm. Suppl.* 56, 127-137
- 15 Monsonego, A. and Weiner, H.L. (2003) Immunotherapeutic approaches to Alzheimer's disease. *Science* 302, 834-838
- 16 Weinreb, R.N. and Levin, L.A. (1999) Is neuroprotection a viable therapy for glaucoma? *Arch. Ophthalmol.* 117, 1540-1544
- 17 Schwartz, M. and Yoles, E. (2000) Neuroprotection: a new treatment modality for glaucoma? *Curr. Opin. Ophthalmol.* 11, 107-111
- 18 Moalem, G. et al. (1999) Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat. Med.* 5, 49-55
- 19 Schwartz, M. et al. (1999) The remedy may lie in ourselves: prospects for immune cell therapy in central nervous system protection and repair. *J. Mol. Med.* 77, 713-717
- 20 Hauben, E. et al. (2000) Passive or active immunization with myelin basic protein promotes recovery from spinal cord contusion. *J. Neurosci.* 20, 6421-6430

21 Kipnis, J. et al. (2000) T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies. *Proc. Natl. Acad. Sci. U S A* 97, 7446-7451

22 Kipnis, J. et al. (2002) Myelin specific Th1 cells are necessary for post-traumatic protective autoimmunity. *J. Neuroimmunol.* 130, 78-85

23 Butovsky, O. et al. (2001) Morphological aspects of spinal cord autoimmune neuroprotection: colocalization of T cells with B7--2 (CD86) and prevention of cyst formation. *Faseb J.* 15, 1065-1067

24 Barouch, R. and Schwartz, M. (2002) Autoreactive T cells induce neurotrophin production by immune and neural cells in injured rat optic nerve: implications for protective autoimmunity. *Faseb J.* 16, 1304-1306

25 Moalem, G. et al. (2000) Production of neurotrophins by activated T cells: Implications for neuroprotective autoimmunity. *J. Autoimmun.* 20, 6421-6430

26 Lidman, O. et al. (2002) Facial nerve lesion response; strain differences but no involvement of IFN-gamma, STAT4 or STAT6. *Neuroreport* 13, 1589-1593

27 Olsson, T. et al. (2000) Genetic regulation of nerve avulsion-induced spinal cord inflammation. *Ann. N Y Acad. Sci.* 917, 186-196

28 Lundberg, C. et al. (2001) Neurodegeneration and glial activation patterns after mechanical nerve injury are differentially regulated by non-MHC genes in congenic inbred rat strains. *J. Comp. Neurol.* 431, 75-87

29 Hammarberg, H. et al. (2000) Neuroprotection by encephalomyelitis: rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. *J. Neurosci.* 20, 5283-5291

30 Gielen, A. et al. (2003) Increased brain-derived neurotrophic factor expression in white blood cells of relapsing-remitting multiple sclerosis patients. *Scand. J. Immunol.* 57, 493-497

31 Yoles, E. et al. (2001) Protective autoimmunity is a physiological response to CNS trauma. *J. Neurosci.* 21, 3740-3748

32 Kipnis, J. et al. (2002) Neuroprotective autoimmunity: naturally occurring CD4+CD25+ regulatory T cells suppress the ability to withstand injury to the central nervous system. *Proc. Natl. Acad. Sci. U S A* 99, 15620-15625

33 Kipnis, J. et al. (2004) Dual effect of CD4+CD25+ regulatory T cells in neurodegeneration: Pro- and anti-inflammatory cytokines determine microglial activity. *Proc. Natl. Acad. Sci. U S A* In Press

34 Schwartz, M. and Kipnis, J. (2002) Multiple sclerosis as a by-product of the failure to sustain protective autoimmunity: a paradigm shift. *Neuroscientist* 8, 405-413

35 Conlon, P. and Steinman, L. (2002) Altered peptide ligands and MS treatment. *Science* 296, 1801-1802; author reply 1801-1802

36 Gold, D.P. et al. (1997) T-cell receptor peptides as immunotherapy for autoimmune disease. *Crit. Rev. Immunol.* 17, 507-510

37 Hafler, D.A. and Weiner, H.L. (1989) MS: a CNS and systemic autoimmune disease. *Immunol. Today* 10, 104-107

38 Smith, K.M. et al. (1997) Pancreatic gene expression in rare cells of thymic medulla: evidence for functional contribution to T cell tolerance. *Int. Immunol.* 9, 1355-1365

39 van den Beemd, R. et al. (2000) Flow cytometric analysis of the Vbeta repertoire in healthy controls. *Cytometry* 40, 336-345

40 Shimizu, J. et al. (1999) Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* 163, 5211-5218

41 McHugh, R.S. et al. (2001) A T cell receptor transgenic model of severe, spontaneous organ-specific autoimmunity. *Eur J Immunol* 31, 2094-2103

42 Monsonego, A. et al. (2003) Beneficial effect of orally administered myelin basic protein in EAE-susceptible Lewis rats in a model of acute CNS degeneration. *J. Autoimmun.* 21, 131-138

43 Frenkel, D. et al. (2003) Nasal vaccination with myelin oligodendrocyte glycoprotein reduces stroke size by inducing IL-10-producing CD4+ T cells. *J. Immunol.* 171, 6549-6555

44 Yoon, D.H. et al. (1999) Therapeutic time window for methylprednisolone in spinal cord injured rat. *Yonsei Med. J.* 40, 313-320

45 Short, D.J. et al. (2000) High dose methylprednisolone in the management of acute spinal cord injury - a systematic review from a clinical perspective. *Spinal Cord* 38, 273-286

46 Bethea, J.R. and Dietrich, W.D. (2002) Targeting the host inflammatory response in traumatic spinal cord injury. *Curr. Opin. Neurol.* 15, 355-360

47 Jones, T.B. et al. (2004) Passive or active immunization with myelin basic protein impairs neurological function and exacerbates neuropathology after spinal cord injury in rats. *J. Neurosci.* 24, 3752-3761

48 Moore, G.R. et al. (1985) Dose-dependency of MBP-induced demyelination in the guinea pig. *J. Neurol. Sci.* 70, 197-205

49 Moore, G.R. et al. (1987) Critical threshold for dose of myelin basic protein in murine autoimmune encephalomyelitis. *J. Neurol. Sci.* 77, 173-184

50 Hauben, E. et al. (2001) Posttraumatic therapeutic vaccination with modified myelin self-antigen prevents complete paralysis while avoiding autoimmune disease. *J. Clin. Invest.* 108, 591-599

51 Huang, D.W. et al. (1999) A therapeutic vaccine approach to stimulate axon regeneration in the adult mammalian spinal cord. *Neuron* 24, 639-647

52 Sicotte, M. et al. (2003) Immunization with myelin or recombinant Nogo-66/MAG in alum promotes axon regeneration and sprouting after corticospinal tract lesions in the spinal cord. *Mol. Cell. Neurosci.* 23, 251-263

53 Muhallab, S. et al. (2002) Differential expression of neurotrophic factors and inflammatory cytokines by myelin basic protein-specific and other recruited T cells infiltrating the central nervous system during experimental autoimmune encephalomyelitis. *Scand. J. Immunol.* 55, 264-273

54 Hofstetter, H.H. et al. (2003) Autoreactive T cells promote post-traumatic healing in the central nervous system. *J. Neuroimmunol.* 134, 25-34

55 Rutkowski, M.D. et al. (2004) Presence of spinal B7.2 (CD86) but not B7.1 (CD80) co-stimulatory molecules following peripheral nerve injury: role of nondestructive immunity in neuropathic pain. *J. Neuroimmunol.* 146, 94-98

56 Merkler, D. et al. (2003) Rapid induction of autoantibodies against Nogo-A and MOG in the absence of an encephalitogenic T cell response: implication for immunotherapeutic approaches in neurological diseases. *Faseb J.* 17, 2275-2277

57 Byram, S.C. et al. (2004) CD4-positive T cell-mediated neuroprotection requires dual compartment antigen presentation. *J Neurosci* 24, 4333-4339

58 Mizrahi, T. et al. (2002) The tissue-specific self-pathogen is the protective self-antigen: the case of uveitis. *J. Immunol.* 169, 5971-5977

59 Sela, M. (1999) Specific vaccines against autoimmune diseases. *C R Acad. Sci. III* 322, 933-938

60 Duda, P.W. et al. (2000) Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. *J. Clin. Invest.* 105, 967-976

61 Schori, H. et al. (2001) Vaccination for protection of retinal ganglion cells against death from glutamate cytotoxicity and ocular hypertension: Implications for glaucoma. *Proc. Natl. Acad. Sci. U S A* 98, 3398-3403

62 Angelov, D.N. et al. (2003) Therapeutic vaccine for acute and chronic motor neuron diseases: implications for amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. U S A* 100, 4790-4795

63 Bakalash, S. et al. (2003) Antigenic specificity of immunoprotective therapeutic vaccination for glaucoma. *Invest Ophthalmol Vis Sci* 44, 3374-3381

64 Benner, E. et al. (2004) Therapeutic Immunization Protects Dopaminergic Neurons in a Mouse Model of Parkinson's Disease. *Proc. Natl. Acad. Sci. U S A*, In press

65 Kipnis, J. et al. (2003) Therapeutic vaccination for closed head injury. *J. Neurotrauma* 20, 559-569

66 Dodel, R.C. et al. (2003) Immunotherapy for Alzheimer's disease. *Lancet Neurol.* 2, 215-220

67 Vickers, J.C. (2002) A vaccine against Alzheimer's disease: developments to date. *Drugs Aging* 19, 487-494

68 Monsonego, A. et al. (2003) Increased T cell reactivity to amyloid beta protein in older humans and patients with Alzheimer disease. *J. Clin. Invest.* 112, 415-422

69 Kipnis, J. et al. (2004) Low-dose gamma-irradiation promotes survival of injured neurons in the central nervous system via homeostasis-driven proliferation of T cells. *Eur. J. Neurosci.* 19, 1191-1198

70 Dummer, W. et al. (2002) T cell homeostatic proliferation elicits effective antitumor autoimmunity. *J. Clin. Invest.* 110, 185-192

71 Ma, J. et al. (2003) Anti-tumor T cell response and protective immunity in mice that received sublethal irradiation and immune reconstitution. *Eur. J. Immunol.* 33, 2123-2132

72 Elflein, K. et al. (2003) Rapid recovery from T lymphopenia by CD28 superagonist therapy. *Blood* 102, 1764-1770

73 Gudmundsdottir, H. and Turka, L.A. (2001) A closer look at homeostatic proliferation of CD4+ T cells: costimulatory requirements and role in memory formation. *J. Immunol.* 167, 3699-3707

Figure Legends

Figure 1. *Similarity between acute and chronic CNS injuries: Pharmacological versus immune-based intervention.* Neurodegenerative conditions in the CNS, both acute and chronic, share similar mediators of secondary degeneration (causing neuronal death). These include impaired blood supply, decreased production of growth factors, activated glial cells, increased free radicals, and neurotransmitters which, having escaped regulatory control, are present in cytotoxic excess of their physiological concentrations. In view of the commonality of these mediators in acute and chronic neurodegenerative conditions, we propose that similar therapeutic interventions might be effective in both. Whereas pharmacological intervention operates by highly specific targeting of a single mediator, necessitating a cocktail of therapies, adaptive immunity operates by targeting with lower specificity a variety of toxic mediators, and is therefore significantly more beneficial. Adaptive immunity can be boosted, moreover, either by directly increasing the number of autoimmune effector T cells or by down-regulating Treg (allowing more rapid and more efficient activation of autoimmune T cells).

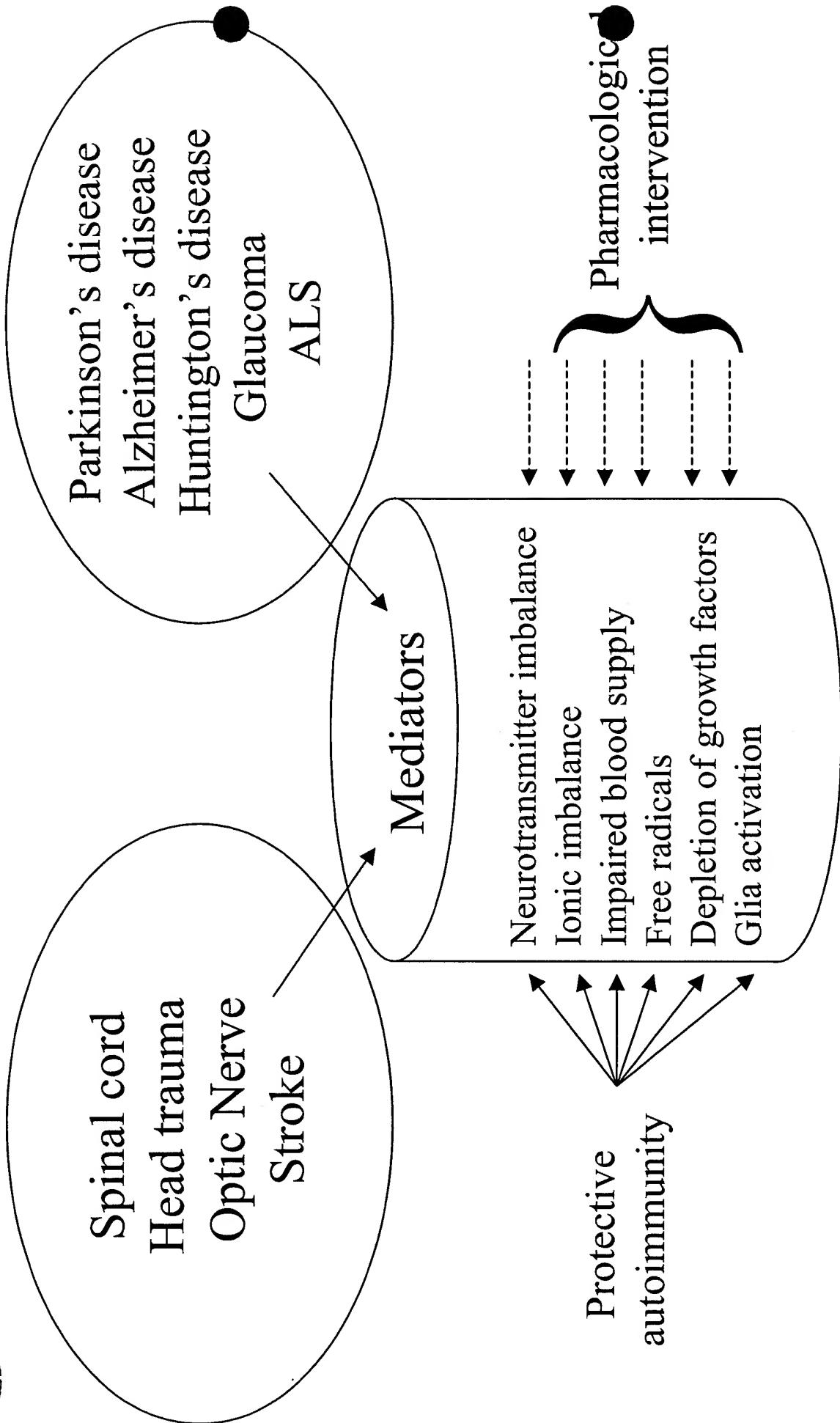
Figure 2. *Accessibility of effector T cells as a function of the number of regulatory T cells and time after triggering by a signal.* Theoretically, the numbers of Treg in a specific autoreactive T cell clone can range from zero (in animals devoid of Treg) to high. The Teff/Treg ratio will determine the number of Teff that become accessible upon triggering (by injury, vaccination, etc.). In the presence of small amounts of Treg the activation of Teff will be high and immediate, whereas in the presence of abundant Treg, Teff activation will be delayed and then will be uncontrolled. Arrows indicate the hypothetical case in resistant and susceptible animal strains, which might explain the paradoxical situation of in susceptible strains, where low spontaneous

ability to withstand injurious conditions coupled with uncontrolled autoimmunity leads to autoimmune disease.

CNS neurodegenerative conditions

Acute injury

Chronic neurodegeneration



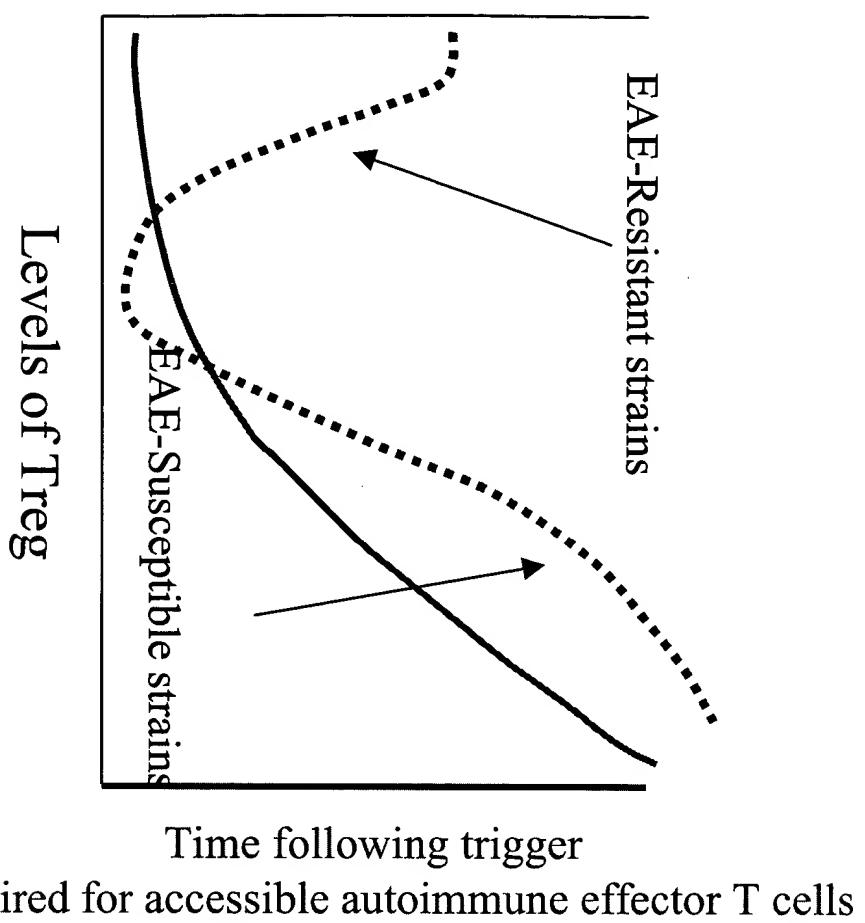
A single immunological intervention
fights off all mediators

Drugs specific to each
mediator

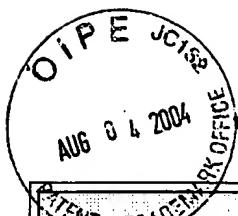




Accessible autoimmune effector T cells upon trigger



Required for accessible autoimmune effector T cells



REVIEW ARTICLE

MECHANISMS OF DISEASE

Apoptosis and Caspases in Neurodegenerative Diseases

Robert M. Friedlander, M.D.

ACUTE AND CHRONIC NEURODEGENERATIVE DISEASES ARE ILLNESSES associated with high morbidity and mortality, and few or no effective options are available for their treatment. A characteristic of many neurodegenerative diseases—which include stroke, brain trauma, spinal cord injury, amyotrophic lateral sclerosis (ALS), Huntington's disease, Alzheimer's disease, and Parkinson's disease—is neuronal-cell death.¹ Given that central nervous system tissue has very limited, if any, regenerative capacity, it is of utmost importance to limit the damage caused by neuronal death.²⁻⁵ During the past decade, considerable progress has been made in understanding the process of cell death.⁶ In this article, I review the causes and mechanisms of neuronal-cell death, especially as it pertains to the caspase family of proteases associated with cell death. I will review evidence linking specific cell-death pathways to neurologic diseases and discuss how knowledge of the mechanisms of cell death has led to novel therapeutic strategies.

TYPES OF CELL DEATH

Cell death occurs by necrosis or apoptosis.⁷⁻⁹ These two mechanisms have distinct histologic and biochemical signatures. In necrosis, the stimulus of death (e.g., ischemia) is itself often the direct cause of the demise of the cell. In apoptosis, by contrast, the stimulus of death activates a cascade of events that orchestrate the destruction of the cell. Unlike necrosis, which is a pathologic process, apoptosis is part of normal development (physiologic apoptosis); however, it also occurs in a variety of diseases (aberrant apoptosis).

NECROSIS

Necrotic cell death in the central nervous system follows acute ischemia or traumatic injury to the brain or spinal cord.^{10,11} It occurs in areas that are most severely affected by abrupt biochemical collapse, which leads to the generation of free radicals and excitotoxins (e.g., glutamate, cytotoxic cytokines, and calcium). The histologic features of necrotic cell death are mitochondrial and nuclear swelling, dissolution of organelles, and condensation of chromatin around the nucleus. These events are followed by the rupture of nuclear and cytoplasmic membranes and the degradation of DNA by random enzymatic cuts in the molecule.^{9,12} Given these mechanisms and the rapidity with which the process occurs, necrotic cell death is extremely difficult to treat or prevent.

APOPTOSIS

Apoptotic cell death, also known as programmed cell death, can be a feature of both acute and chronic neurologic diseases.^{1,9,13} After acute insults, apoptosis occurs in areas that are not severely affected by the injury. For example, after ischemia, there is ne-

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crotic cell death in the core of the lesion, where hypoxia is most severe, and apoptosis occurs in the penumbra, where collateral blood flow reduces the degree of hypoxia (Fig. 1).^{10,14-16} Apoptotic death is also a component of the lesion that appears after brain or spinal cord injury.^{11,17-20} In chronic neurodegenerative diseases, it is the predominant form of cell death.²¹⁻²³

In apoptosis, a biochemical cascade activates proteases that destroy molecules that are required for cell survival and others that mediate a program of cell suicide. During the process, the cytoplasm condenses, mitochondria and ribosomes aggregate, the nucleus condenses, and chromatin aggregates. After its death, the cell fragments into "apoptotic bodies," and chromosomal DNA is enzymatically cleaved to 180-bp internucleosomal fragments. Other features of apoptosis are a reduction in the membrane potential of the mitochondria, intracellular

acidification, generation of free radicals, and externalization of phosphatidylserine residues.^{6,7,12,24,25}

MECHANISMS OF PROGRAMMED CELL DEATH

The rational development of target-based strategies for the treatment of diseases in which apoptosis is prominent requires an understanding of the molecular mechanisms of programmed cell death. As recently as 10 years ago, the mediators of this process were for the most part unknown. Beginning in 1993, a series of seminal studies of the nematode *Caenorhabditis elegans* identified several genes that control cell death.²⁶ In this worm, four genes are required for the orderly execution of the developmental apoptotic program. The *ced-3*, *ced-4*, and *egl-1* genes mediate cell death, and worms that have lost the function of these genes harbor extra cells.^{27,28} By contrast, *ced-9*-deficient worms have diffuse apoptotic cell death, indicating that this gene functions as an inhibitor of apoptosis. Metazoan homologues of *ced-3* (caspases), *ced-4* (*Apaf-1*), *ced-9* (*Bcl-2*), and *egl-1* (*BH3-only proteins*) have been identified.^{27,29-32}

CASPASE FAMILY

The major executioners in the apoptotic program are proteases known as caspases (cysteine-dependent, aspartate-specific proteases).^{6,33} Caspases are cysteine proteases that are homologous to the nematode *ced-3* gene product. The interleukin-1 β -converting enzyme (also known as caspase 1), the founding member of the caspase family in vertebrates, was identified by its homology to *ced-3*.^{27,29} Thus far, 14 members of the caspase family have been identified, 11 of which are present in humans.²⁷ Caspases directly and indirectly orchestrate the morphologic changes of the cell during apoptosis.

Caspases exist as latent precursors, which, when activated, initiate the death program by destroying key components of the cellular infrastructure and activating factors that mediate damage to the cells. Procaspses are composed of p10 and p20 subunits and an N-terminal recruitment domain. Active caspases are heterotetramers consisting of two p10 and two p20 subunits derived from two pro-caspase molecules (Fig. 2). Caspases have been categorized into upstream initiators and downstream executioners. Upstream caspases are activated by the cell-death signal (e.g., tumor necrosis factor α

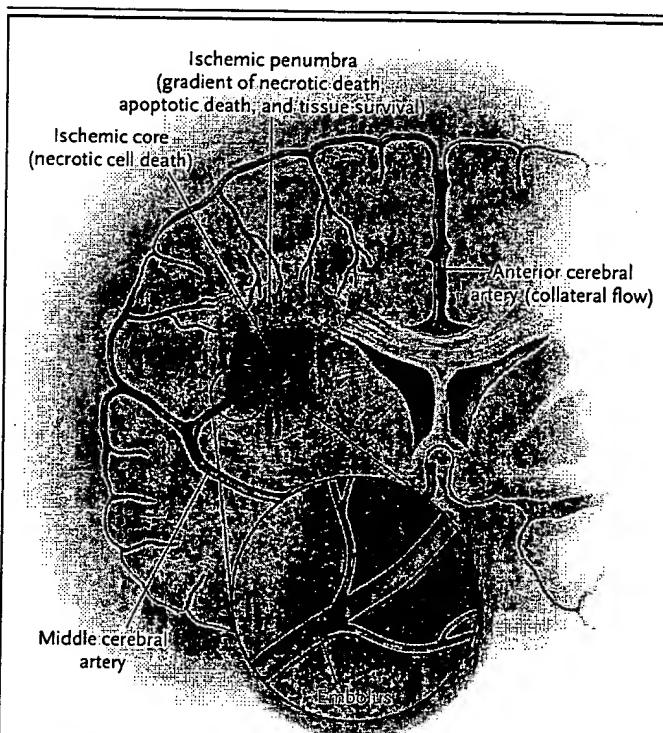


Figure 1. An Embolus in the Bifurcation of the Middle Cerebral Artery. The territory perfused by this artery and areas with little or no collateral flow are subjected to extreme hypoxia and necrotic cell death. In the penumbra, where there is some degree of collateral blood flow, a gradient of its perfusion establishes a threshold among necrotic cell death, apoptotic cell death, and tissue survival.

[TNF- α]) and have a long N-terminal prodomain that regulates their activation.^{6,34} These upstream caspases activate downstream caspases, which directly mediate the events leading to the demise of the cell. Downstream caspases have a short N-terminal prodomain.

A critical aspect of caspase-mediated cell death lies in the events regulating the activation of initiator caspases. Upstream caspases may be subdivided into two groups, according to the molecules modulating their activation. Procaspses 1, 2, 4, 5, 9, 11, 12, and 13 have a long N-terminal prodomain called the caspase-recruiting domain (CARD). Caspases 8 and 10 have a long N-terminal prodomain called the death-effector domain (DED). A regulating molecule is required for specific binding to the CARD/DED domain, which results in caspase activation. These molecules are caspase-specific and trigger-specific. For example, after the binding of TNF- α to its receptor, the TNF receptor binds to the DED molecule that mediates caspase 8 activation. Of the caspases with a long prodomain, caspases 2, 8, 9, and 10 are initiators of apoptosis and caspases 1, 4, 5, 11, 12, and 13 are involved in cytokine activation.³⁴ There is mounting evidence that in addition to its role in inflammation, caspase 1 is also an important upstream caspase.^{18,35-45}

Once upstream caspases are activated in an amplifying cascade, they activate the executioner caspases downstream.^{6,34,46} Of these caspases with a short prodomain, caspases 3, 6, and 7 are effectors of apoptosis and caspase 14 is involved in cytokine maturation. Executioner caspases mediate cell death by two main mechanisms: destruction and activation. The systematic destruction of key cellular substrates is crucial. The death process begins its terminal phase when executioner caspases activate the machinery that degrades DNA.^{25,47-49}

Caspases are also regulated at the transcriptional level. Transcriptional up-regulation of caspases occurs in chronic neurologic diseases such as ALS and Huntington's disease, as well as in acute neurologic diseases such as stroke,^{35,38,50,51} which indicates that the degree of activation and the number of caspase molecules within the cell determine the level of caspase activity.

ROLE OF THE Bcl-2 FAMILY IN REGULATING RELEASE OF MITOCHONDRIAL CYTOCHROME C

Cytochrome c is a member of the mitochondrial electron-transport chain that is required for the generation of ATP. In addition to its role in cellular

energetics, cytochrome c is an important trigger of the caspase cascade. Cytochrome c-mediated activation of cell-death pathways occurs if cytochrome c is released from the mitochondria into the cytoplasm. In the cytoplasm, cytochrome c binds to Apaf-1 to form the apoptosome—a molecular complex consisting of cytochrome c, Apaf-1, ATP, and procaspase 9. The apoptosome activates caspase 9,^{30,52} an upstream initiator of apoptosis. This mechanism makes regulation of the release of cytochrome c a key step in the initiation of apoptosis (Fig. 3).^{6,53}

Members of the Bcl-2 family are proapoptotic or antiapoptotic. The balance between proapoptotic and antiapoptotic signals from the Bcl-2 family has a crucial role in the release of cytochrome c.^{6,54,55} Moreover, members of the caspase family can influence the balance of proapoptotic and antiapoptotic signals from the Bcl-2 family. For example, caspase 8 and caspase 1 cleave Bid, a member of

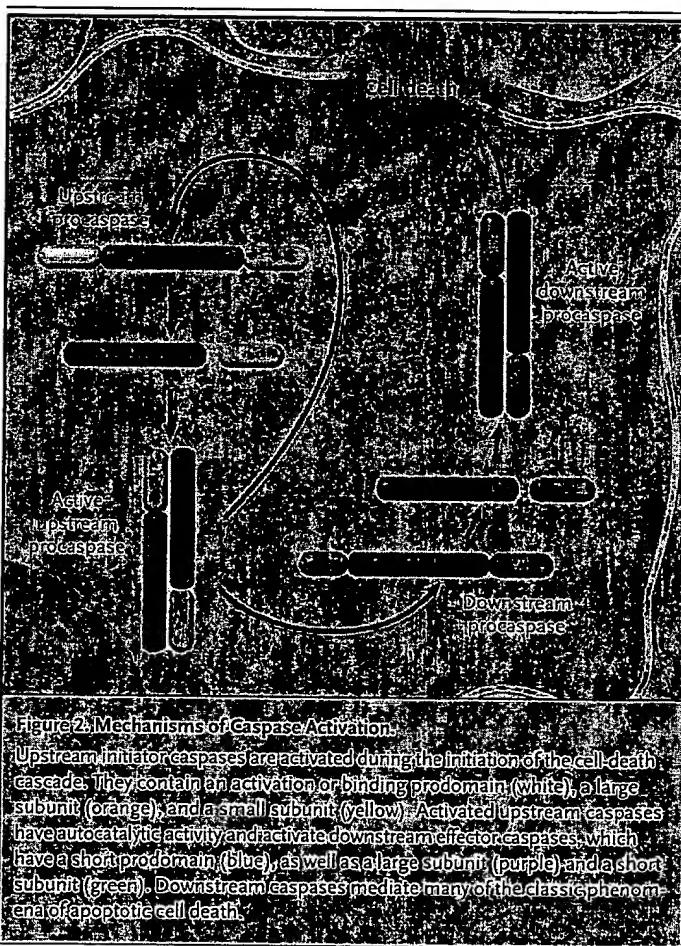
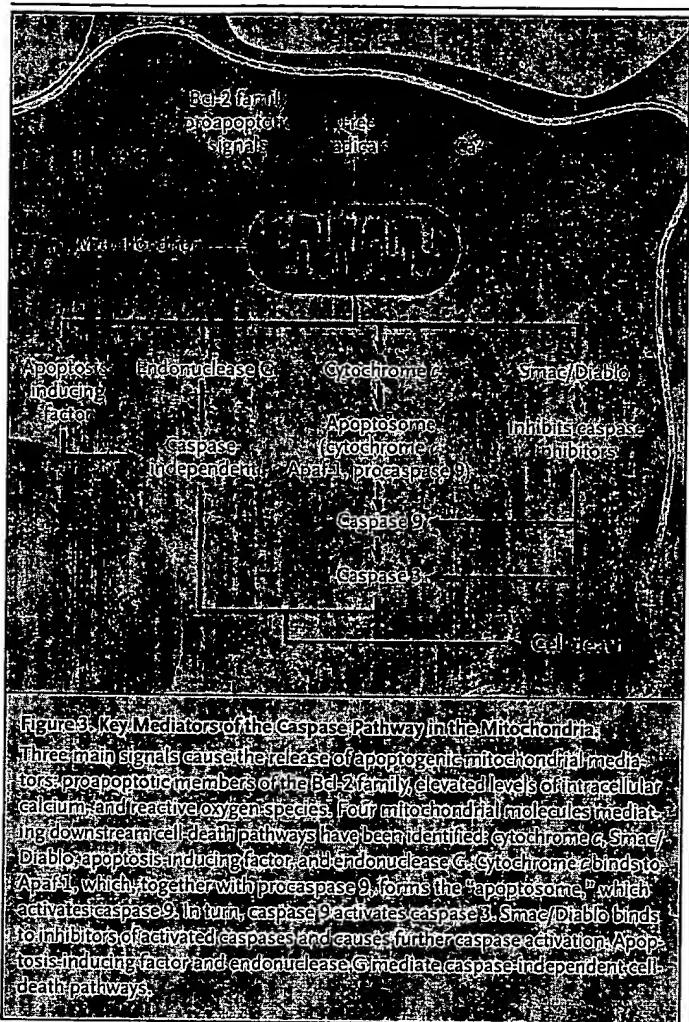


Figure 2. Mechanisms of Caspase Activation.

Upstream initiator caspases are activated during the initiation of the cell-death cascade. They contain an activation or binding prodomain (white), a large subunit (orange), and a small subunit (yellow). Activated upstream caspases have autocatalytic activity and activate downstream effector caspases, which have a short prodomain (blue), as well as a large subunit (purple) and a short subunit (green). Downstream caspases mediate many of the classic phenomena of apoptotic cell death.



the Bcl-2 family, generating a truncated fragment with proapoptotic activity.⁵⁶ In addition to cytochrome *c*, other modulators of cell death within mitochondria are released during the apoptotic process.⁵³

INHIBITORS OF APOPTOSIS

To control aberrant caspase activation, which can kill the cell, additional molecules inhibit caspase-mediated pathways. Among these are proteins known as inhibitors of apoptosis. These inhibitors interact directly with modulators of cell death. For example, the X-linked inhibitor of apoptosis and the neuronal inhibitor of apoptosis are proteins in neurons that directly inhibit caspase 3 activity and protect neurons from ischemic injury.^{34,55,57}

CASPASES IN NEUROLOGIC DISEASES

Caspases have a pivotal role in the progression of a variety of neurologic disorders. Despite the various causes of such disorders, the mechanism of cell death is similar in a broad spectrum of neurologic diseases.^{1,37,58} However, the trigger of aberrant caspase activation in most of these diseases is not well understood. In acute neurologic diseases, both necrosis and caspase-mediated apoptotic cell death occur.^{11,17,36,59,60} By contrast, in chronic neurodegenerative diseases, caspase-mediated apoptotic pathways have the dominant role in mediating cell dysfunction and cell death.^{38,39,61,62} A primary difference between acute and chronic neurologic diseases is the magnitude of the stimulus causing cell death. The greater stimulus in acute diseases results in both necrotic and apoptotic cell death, whereas the milder insults in chronic diseases initiate apoptotic cell death.

ACUTE NEUROLOGIC DISEASES

Ischemic stroke was the first neurologic disease in which the activation of a caspase (caspase 1) was documented.⁴⁴ Moreover, inhibition of caspases reduces tissue damage and allows remarkable neurologic improvement.^{44,63,64} Activation of caspases 1, 3, 8, 9, and 11 and release of cytochrome *c* have been demonstrated in cerebral ischemia,^{41,65-67} and the Bcl-2 family has also been incriminated.^{68,69} Mice that express a dominant-negative caspase 1 construct or that are deficient in caspase 1 or caspase 11 have significant protection from ischemic injury.^{44,65,70} Pharmacologic pretreatment of mice with a broad caspase inhibitor or with semi-selective inhibitors of caspase 1 and caspase 3 or delayed treatment with a caspase 3 inhibitor protect the brain from ischemic injury.^{64,71}

There is a pattern of combined necrotic and apoptotic cell death after ischemic or traumatic injury.^{15,18-20,36,59} In ischemia, necrotic cell death occurs in the core of the infarction, where hypoxia is most severe, and leads to abrupt cessation of energy supply and acute cellular collapse. Conversely, in the ischemic penumbra, the degree of energy deprivation is not as severe, because collateral vessels supply the region with oxygenated blood. In this case, the cell must reach a critical threshold of injury to activate the caspase cascade. Before this threshold is reached, however, a compromise in neuronal energetics can cause cell dysfunction before cell death. What determines the threshold in a

particular cell is unknown. Nevertheless, the existence of the threshold offers an opportunity to rescue cells in the penumbra by reversing the initial neurologic deficit caused by cell dysfunction. Factors that promote survival can raise the threshold, as evidenced in the experiments with caspase inhibition described above and in studies in which the balance among members of the Bcl-2 family was transgenically manipulated.^{68,69} The cerebral tissue protected by modulation of caspase activation is invariably the penumbra.^{44,64,66,68}

CHRONIC NEURODEGENERATIVE DISEASES

Cell death in chronic neurodegenerative diseases often occurs as a result of a mutation in one or several genes. This genetic alteration changes the function of the gene product in a way that has a detrimental effect on the cell. Environmental factors have also been incriminated in chronic neurodegeneration, but the cause of many such disorders remains unknown. I will describe the key role of the caspase family in two diseases, ALS and Huntington's disease. There is evidence suggesting that caspases have a role in Alzheimer's disease, Parkinson's disease, and dementia associated with human immunodeficiency virus infection.^{62,72,73} The cause of the selective death of motor neurons in ALS or of medium-sized spiny neurons in the striatum in Huntington's disease is, for the most part, not understood. This question is the focus of intense investigation.

ALS

ALS is characterized by the progressive and specific loss of motor neurons in the brain, brain stem, and spinal cord.⁷⁴ The average age at onset is 55 years, and the average life expectancy after the clinical onset is 4 years. The only recognized treatment for ALS is riluzole, whose use extends survival by only about three months. Familial and sporadic forms of the disease have been described. The natural history and histologic abnormalities in these two forms of ALS are not distinguishable.

A mutation in the gene encoding superoxide dismutase 1 (SOD1) has been identified in 10 percent of patients with familial ALS.⁷⁵ In transgenic mice expressing the human mutant SOD1 gene, a syndrome develops with many features of ALS, including specific cell death of motor neurons, progressive weakness, and early death.⁷⁶ These mouse models of ALS and other mice with additional ALS-linked mutations in SOD1 are effective tools for the

study of molecular mechanisms and pharmacotherapy for ALS.^{38,67,77} The first evidence of a role of a caspase in a neurodegenerative disease came from experiments in which the "ALS mouse" was cross-bred with a mouse expressing a mutant caspase 1 gene that inhibited caspase 1 in neurons.⁶¹ As compared with mice expressing only the mutant SOD1 transgene, mice expressing both the mutant SOD1 transgene and the mutant caspase 1 transgene had a duration of survival that was greater by 9 percent, and disease progression was slowed by more than 50 percent. Furthermore, intraventricular administration of a broad caspase inhibitor (zVAD-fmk) was neuroprotective and extended survival in the ALS mice by 22 percent.³⁸

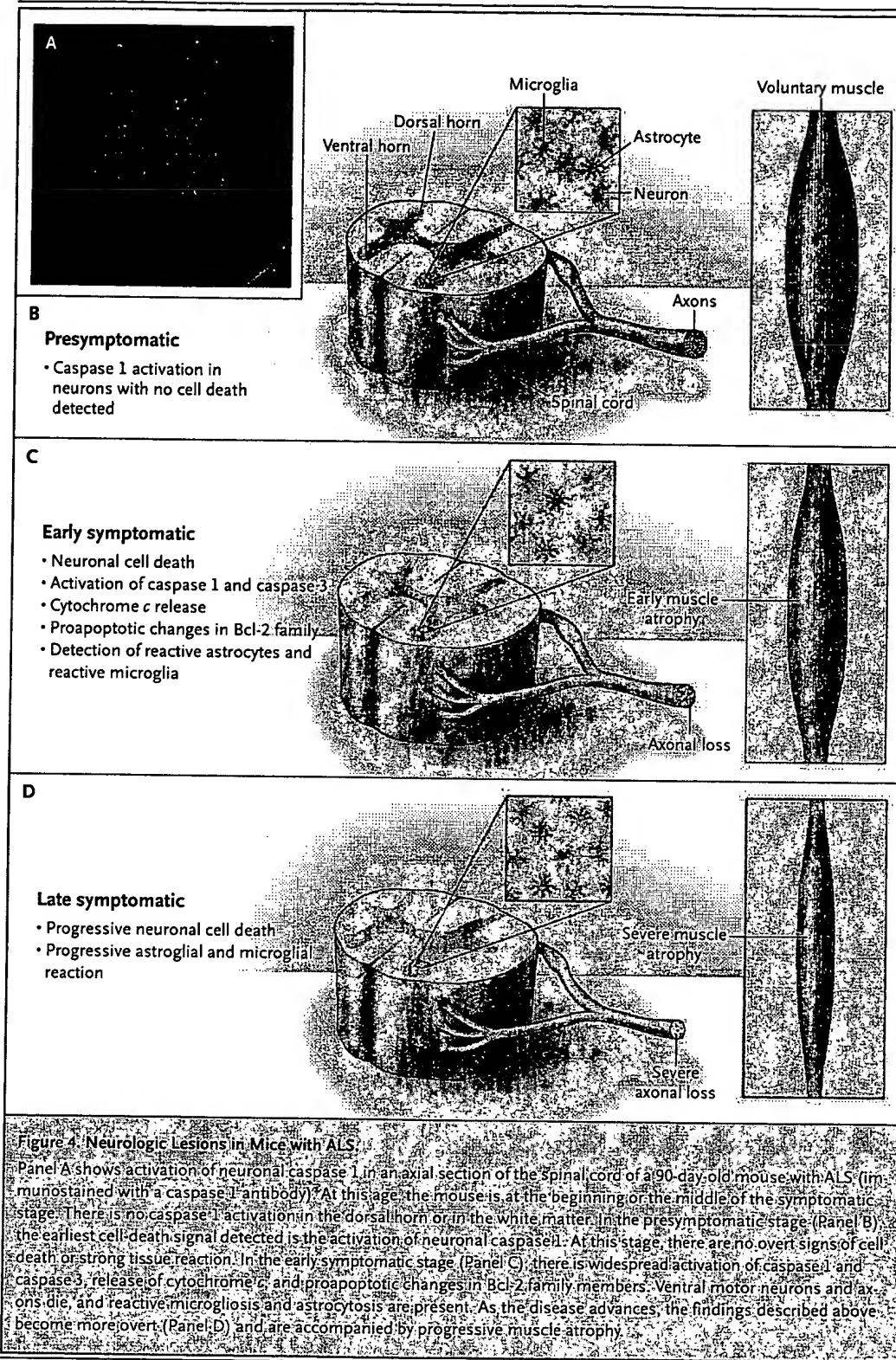
A prolonged period of neuronal caspase activation (especially of caspase 1) was detected in transgenic ALS mice (Fig. 4A).^{38,42,43} As these mice aged, there was progressive transcriptional up-regulation of caspase 1 messenger RNA (mRNA), followed by up-regulation of caspase 3 mRNA (Fig. 4B). Despite treatment of ALS mice with the enzymatic caspase inhibitor zVAD-fmk, transcriptional up-regulation of caspase 1 and caspase 3 was delayed, suggesting that there is a non-cell-autonomous "contagious" apoptotic process in these mice (see below).³⁸ These sequential events are also detected at the level of enzymatic activity.^{38,40,43} The finding of caspase 1 and caspase 3 activation in spinal cord samples from patients with ALS indicates the clinical relevance of these animal models of ALS.^{38,78}

Caspase 9 activation, cytochrome c release, and proapoptotic changes in the Bcl-2 family have also been detected in spinal cords of ALS mice.^{67,79} Moreover, ALS mice bearing a transgenic Bcl-2 gene survive longer than other ALS mice.⁸⁰

Huntington's Disease

Huntington's disease is an autosomal dominant neurodegenerative disorder in which specific cell death occurs in the neostriatum and cortex.^{13,81} Onset usually occurs during the fourth or fifth decade of life, with a mean survival after onset of 15 to 20 years. Huntington's disease is universally fatal, and there is no effective treatment. Symptoms include a characteristic movement disorder (Huntington's chorea), cognitive dysfunction, and psychiatric symptoms. The disease is caused by a mutation encoding an abnormal expansion of CAG-encoded polyglutamine repeats in a protein called huntingtin.⁸²

The discovery of the mutant gene responsible for the disease made it possible to create transgenic



mouse models of it.⁸³ In these mice, apoptotic pathways and newly described cell-death pathways that are neither apoptotic nor necrotic have been demonstrated.^{84,85} One of the earliest events in the pre-symptomatic and early symptomatic stages of the disease is transcriptional up-regulation of the caspase 1 gene.³⁹ This event appears to result from nuclear translocation of N-terminal fragments of mutant huntingtin.⁸⁶ As the disease progresses, the caspase 3 gene is transcriptionally up-regulated, and the protein is activated.³⁵ Activation of caspase 8 and caspase 9 and release of cytochrome *c* have also been demonstrated in Huntington's disease.^{87,88}

Evidence is beginning to accumulate of both a toxic effect of huntingtin fragments and depletion of huntingtin in Huntington's disease.^{35,39,89-91} Huntingtin is a substrate for caspase 1 and caspase 3.^{92,93} As the disease progresses, increased caspase-mediated cleavage of huntingtin increases the generation of huntingtin fragments and depletes wild-type huntingtin (Fig. 5).³⁹ It appears that some features of Huntington's disease result from the depletion of this protein.⁹⁴

Neuronal dysfunction caused by the down-regulation of receptors that bind important neurotransmitters is another important feature of Huntington's disease.⁹⁵ We know that this down-regulation of receptors is, at least in part, a caspase-mediated event, since the inhibition of caspase also inhibits receptor down-regulation.³⁹ This evidence suggests that caspases are mediators not only of cell death but also of cell dysfunction.

Several of the findings in mouse models of Huntington's disease have also been demonstrated in human striatal brain tissue, including activation of caspases 1, 3, 8, and 9 and release of cytochrome *c*.^{39,87,88} Transgenic mice have been used as a tool for evaluating and demonstrating the efficacy of caspase inhibitors, creatine, and minocycline in an animal model of Huntington's disease.^{35,39,85}

MINOCYCLINE

Minocycline is a second-generation tetracycline with remarkable neuroprotective properties. Because it inhibits the production of nitric oxide by the inducible form of nitric oxide synthetase, minocycline was evaluated in experimental models of cerebral ischemia. Minocycline significantly reduced the severity of ischemia-induced tissue injury and neurologic dysfunction.^{50,51} Along with the neuroprotection it provided, minocycline inhibited the

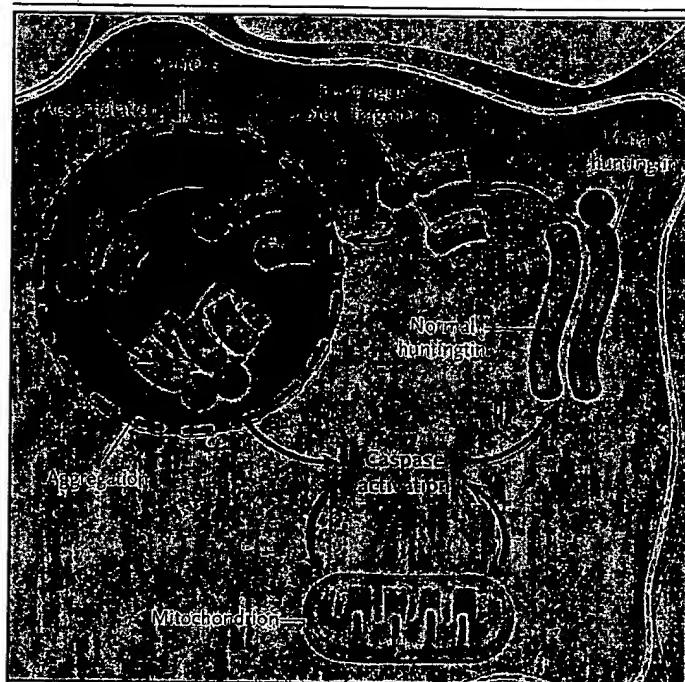


Figure 5. Huntington's Disease.

Neurons of patients with Huntington's disease contain one copy of the wild-type huntingtin allele (producing orange protein) and one copy of the mutant allele (producing orange and blue protein). Possibly as part of the normal proteolysis of huntingtin, an N-terminal fragment is generated. Mutant N-terminal fragments accumulate and aggregate forming neuronal intranuclear inclusions. Nuclear translocation of mutant N-terminal fragments up-regulates transcription of caspase 1. As the disease progresses, caspase 1 activates caspase 3. Caspase 1 and caspase 3 cleave huntingtin, producing N-terminal fragments and resulting in the depletion of huntingtin. As the disease progresses further, Bid is activated, releasing cytochrome *c*. Assembly of the apoptosome activates caspase 9 and caspase 3. Progressive caspase activation leads to neuronal dysfunction and cell death.

ischemia-induced up-regulation of nitric oxide synthase, caspase 1, and reactive microgliosis.⁹⁶ Neuroprotection by minocycline has also been observed in mouse models of Huntington's disease, ALS, brain injury, Parkinson's disease, and multiple sclerosis.^{35,67,97} The primary mechanism of action of minocycline is the direct inhibition of the release of cytochrome *c*; secondarily, it inhibits downstream events related to cell death — in particular, the activation of caspase 3.⁶⁷ It is not clear whether minocycline inhibits reactive microgliosis or the production of nitric oxide synthase directly or by a secondary process that follows the inhibition of cytochrome *c* release. Minocycline is orally bioavailable, crosses the blood-brain barrier, and has a proven safety record in humans. It is being eval-

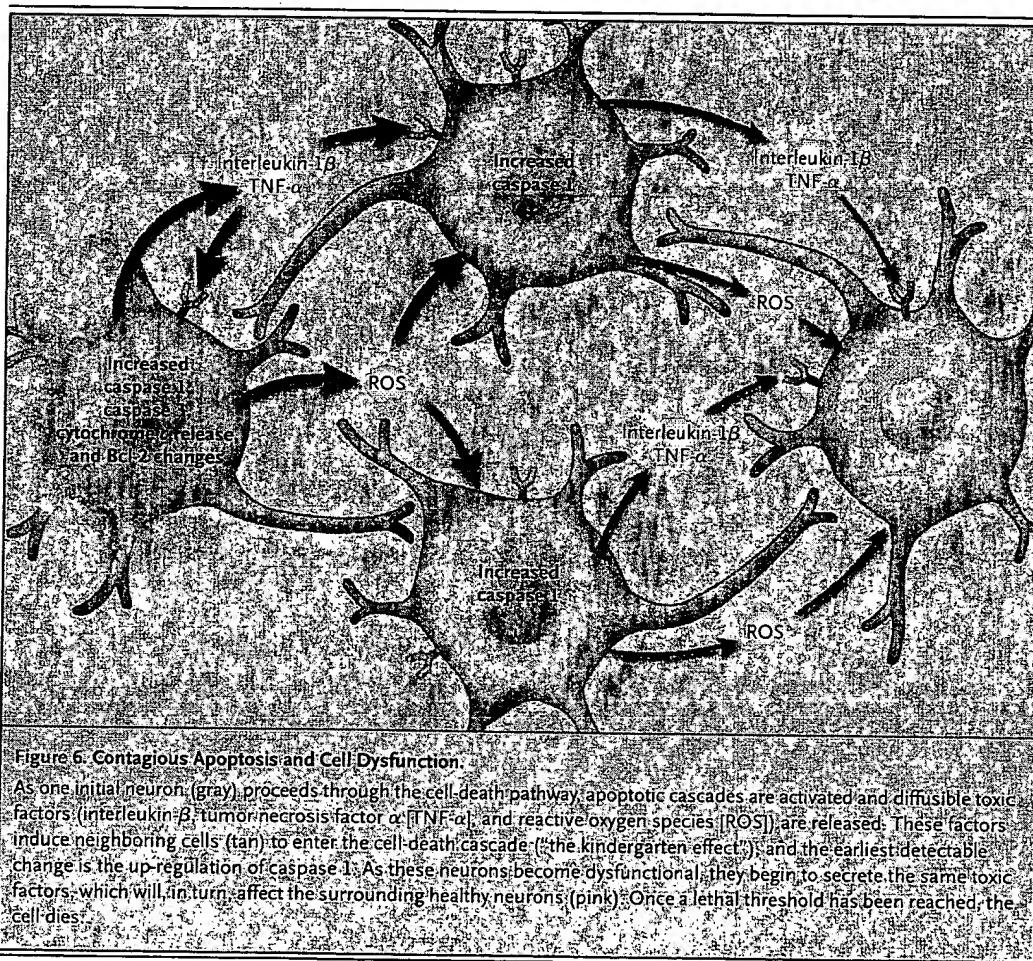
ated in clinical trials in patients with Huntington's disease and ALS.

CONTAGIOUS APOPTOSIS ("THE KINDERGARTEN EFFECT")

The process of cell death in one cell can affect the dynamics of cell death in neighboring cells.³⁸ Factors generated by cells as they die and after they die are detrimental to neighboring cells. Neighboring cells are exposed to triggering factors that are similar to those that affect a cell that is dying. For example, during a stroke, a neuron exposed to an ischemic environment triggers the cell-death cascade and produces interleukin-1 β , TNF- α , and free radicals that play a part in the cell's own demise.¹ These diffusible factors affect neighboring neurons that have been similarly exposed to ischemia. Since there

is a gradient of ischemia, neurons that might not have died as the result of the ischemic insult alone die from a combination of exposure to a sublethal ischemic environment and the diffusible toxic factors generated by their dying neighbors.

This phenomenon also occurs in chronic neurodegenerative diseases. For example, in ALS mediated by mutant SOD1, the mutant SOD1 protein initiates the cell-death cascade in one particular motor neuron. As the neuron progresses through the cascade and eventually dies, it releases proapoptotic factors that affect neighboring cells.³⁸ Since these cells have the same genetic predisposition as their dying neighbor, such factors might induce them, too, to initiate the cell-death cascade (Fig. 6). From a therapeutic standpoint, this concept is important, because an inhibitor of apoptosis not only will slow the process of cell death in one particular cell, but



is also likely to inhibit the production of the diffusible toxic factors that might initiate the cell-death cascade in a neighboring cell.

CHRONIC CASPASE ACTIVATION AND CELL DYSFUNCTION

Apoptotic cell death in the ischemic penumbra results from massive cytotoxic activation of cell-death pathways, whereas in chronic neurodegenerative diseases, weaker stimuli of cell death cause sublethal activation of caspase. Chronic, sublethal activation of caspase appears to mediate cell dysfunction, which precedes cell death.^{38,40} Cell dysfunction of substantial magnitude, occurring before cell death, might result in symptomatic disease. Given that caspases may be active in individual neurons for a long period (potentially weeks to months), inhibition of caspase in these circumstances could reduce cell dysfunction and delay cell death.³⁹ In acute diseases, a delayed wave of cell death can be detected up to one month after the initial injury.^{19,98} Given

the chronic nature of caspase activation, caspase inhibition is a plausible approach to the treatment of neurologic diseases.

CONCLUSIONS

During the past several years, our understanding of the mechanisms mediating cell death in neurologic diseases has improved considerably. The fact that activation of these pathways is a feature of a broad range of neurologic diseases makes them important and attractive therapeutic targets. Pharmaceutical companies are actively searching for compounds that inhibit these pathways. The first clinical trials of an inhibitor of apoptosis (minocycline) for neurodegenerative disorders (Huntington's disease and ALS) are in progress.^{35,38} It is likely that in the next several years, additional inhibitors of apoptosis will become part of the everyday armamentarium of clinicians who are treating neurologic diseases that involve caspase-mediated cell dysfunction and cell death.

REFERENCES

- Yuan J, Yankner BA. Apoptosis in the nervous system. *Nature* 2000;407:802-9.
- Rossi F, Cattaneo E. Neural stem cell therapy for neurological diseases: dreams and reality. *Nat Rev Neurosci* 2002;3:401-9.
- Kuhn HG, Palmer TD, Fuchs E. Adult neurogenesis: a compensatory mechanism for neuronal damage. *Eur Arch Psychiatry Clin Neurosci* 2001;251:152-8.
- Eriksson PS, Perfilieva E, Bjork-Eriksson T, et al. Neurogenesis in the adult human hippocampus. *Nat Med* 1998;4:1313-7.
- Liu J, Solway K, Messing RO, Sharp FR. Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J Neurosci* 1998;18:7768-78.
- Hengartner MO. The biochemistry of apoptosis. *Nature* 2000;407:770-6.
- Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251-306.
- Kanduc D, Mittelman A, Serpico, R, et al. Cell death: apoptosis versus necrosis. *Int J Oncol* 2002;21:165-70.
- Martin LJ. Neuronal cell death in nervous system development, disease, and injury. *Int J Mol Med* 2001;7:455-78.
- Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 1993;24:2002-9.
- Emery E, Aldana P, Bunge MB, et al. Apoptosis after traumatic human spinal cord injury. *J Neurosurg* 1998;89:911-20.
- Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972;26:239-57.
- Martin JB. Molecular basis of the neurodegenerative disorders. *N Engl J Med* 1999;340:1970-80. [Erratum, *N Engl J Med* 1999;341:1407.]
- Charriaut-Marlangue C, Margail I, Represa A, Popovici T, Plotkine M, Ben-Ari Y. Apoptosis and necrosis after reversible focal ischemia: an *in situ* DNA fragmentation analysis. *J Cereb Blood Flow Metab* 1996;16:186-94.
- Li Y, Chopp M, Jiang N, Zaloga C. *In situ* detection of DNA fragmentation after focal cerebral ischemia in mice. *Brain Res Mol Brain Res* 1995;28:164-8.
- MacManus JP, Buchan AM, Hill IE, Rasquinha I, Preston B. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci Lett* 1993;164:89-92.
- Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Beattie MS. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nat Med* 1997;3:73-6. [Erratum, *Nat Med* 1997;3:240.]
- Li M, Ona VO, Chen M, et al. Functional role and therapeutic implications of neuronal caspase-1 and -3 in a mouse model of traumatic spinal cord injury. *Neuroscience* 2000;99:333-42.
- Liu XZ, Xu XM, Hu R, et al. Neuronal and glial apoptosis after traumatic spinal cord injury. *J Neurosci* 1997;17:5395-406.
- Rink A, Fung KM, Trojanowski JQ, Lee VM, Neugebauer E, McIntosh TK. Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. *Am J Pathol* 1995;147:1575-83.
- Thomas LB, Gates DJ, Richfield EK, O'Brien TF, Schweitzer JB, Steindler DA. DNA end labeling (TUNEL) in Huntington's disease and other neuropathological conditions. *Exp Neurol* 1995;133:265-72.
- Troost D, Aten J, Morsink F, de Jong JM. Apoptosis in amyotrophic lateral sclerosis is not restricted to motor neurons: Bcl-2 expression is increased in unaffected post-central gyrus. *Neuropathol Appl Neurobiol* 1995;21:498-504.
- Smale G, Nichols NR, Brady DR, Finch CE, Horton WE Jr. Evidence for apoptotic cell death in Alzheimer's disease. *Exp Neurol* 1995;133:225-30.
- Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* 1980;284:555-6.
- Liu X, Zou H, Slaughter C, Wang X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 1997;89:175-84.
- Horvitz HR. Genetic control of programmed cell death in the nematode *Ceenorhabditis elegans*. *Cancer Res* 1999;59:Suppl:1701s-1706s.
- Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* 1993;75:641-52.
- Yuan J, Horvitz HR. The *Ceenorhabditis elegans* genes *ced-3* and *ced-4* act cell autonomously to cause programmed cell death. *Dev Biol* 1990;138:33-41.
- Miura M, Zhu H, Rotello R, Hartwieg EA, Yuan J. Induction of apoptosis in fibroblasts

by IL-1 beta-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene ced-3. *Cell* 1993;75:653-60.

30. Li P, Nijhawan D, Budihardjo I, et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997;91:479-89.
31. Hengartner MO, Horvitz HR. *C. elegans* cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* 1994;76:665-76.
32. Conradt B, Horvitz HR. The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. *Cell* 1998;93:519-29.
33. Alnemri ES, Livingston DJ, Nicholson DW, et al. Human ICE/CED-3 protease nomenclature. *Cell* 1996;87:171.
34. Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* 2002;9:459-70.
35. Chen M, Ona VO, Li M, et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat Med* 2000;6:797-801.
36. Fink KB, Andrews LJ, Butler WE, et al. Reduction of post-traumatic brain injury and free radical production by inhibition of the caspase-1 cascade. *Neuroscience* 1999;94:1213-8.
37. Friedlander RM, Yuan J. ICE, neuronal apoptosis and neurodegeneration. *Cell Death Differ* 1998;5:823-31.
38. Li M, Ona VO, Guegan C, et al. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science* 2000;288:335-9.
39. Ona VO, Li M, Vonsattel JP, et al. Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. *Nature* 1999;399:263-7.
40. Pasinelli P, Housewear MK, Brown RH Jr, Cleveland DW. Caspase-1 and -3 are sequentially activated in motor neuron death in Cu/Zn superoxide dismutase-mediated familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 2000;97:13901-6.
41. Rabuffetti M, Sciorati C, Tarozzo G, Clementi E, Manfredi AA, Beltramo M. Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-chloromethyl ketone induces long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines. *J Neurosci* 2000;20:4398-404.
42. Sun W, Funakoshi H, Nakamura T. Overexpression of HGF retards disease progression and prolongs life span in a transgenic mouse model of ALS. *J Neurosci* 2002;22:6537-48.
43. Vukosavic S, Stefanis L, Jackson-Lewis V, et al. Delaying caspase activation by Bcl-2: a clue to disease retardation in a transgenic mouse model of amyotrophic lateral sclerosis. *J Neurosci* 2000;20:9119-25.
44. Friedlander RM, Gagliardini V, Hara H, et al. Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. *J Exp Med* 1997;185:933-40.
45. Enari M, Talanian RV, Wong WW, Nagata S. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* 1996;380:723-6.
46. Boucher-Hayes L, Martin SJ. CARD games in apoptosis and immunity. *EMBO Rep* 2002;3:616-21.
47. Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 1998;391:96-9.
48. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 1998;391:43-50. [Erratum, *Nature* 1998;393:396.]
49. Liu X, Li P, Widlak P, et al. The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. *Proc Natl Acad Sci U S A* 1998;95:8461-6.
50. Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc Natl Acad Sci U S A* 1999;96:13496-500.
51. Yrjanheikki J, Keinanen R, Pellikka M, Hokfelt T, Koistinaho J. Tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia. *Proc Natl Acad Sci U S A* 1998;95:15769-74.
52. Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996;86:147-57.
53. Wang X. The expanding role of mitochondria in apoptosis. *Genes Dev* 2001;15:2922-33.
54. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999;13:1899-911.
55. Deveraux QL, Schendel SL, Reed JC. Antiapoptotic proteins: the bcl-2 and inhibitor of apoptosis protein families. *Cardiol Clin* 2001;19:57-74.
56. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998;94:491-501.
57. Xu D, Bureau Y, McIntyre DC, et al. Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. *J Neurosci* 1999;19:5026-33.
58. Troy CM, Salvesen GS. Caspases on the brain. *J Neurosci Res* 2002;69:145-50.
59. Yakovlev AG, Knoblauch SM, Fan L, Fox GB, Goodnight R, Faden AI. Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. *J Neurosci* 1997;17:7415-24.
60. Springer JE, Azbill RD, Knapp PE. Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury. *Nat Med* 1999;5:943-6.
61. Friedlander RM, Brown RH, Gagliardini V, Wang J, Yuan J. Inhibition of ICE slows ALS in mice. *Nature* 1997;388:31. [Erratum, *Nature* 1998;392:560.]
62. Gervais FG, Xu D, Robertson GS, et al. Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. *Cell* 1999;97:395-406.
63. Loddick SA, MacKenzie A, Rothwell NJ. An ICB inhibitor, z-VAD-DCB attenuates ischaemic brain damage in the rat. *Neuroreport* 1996;7:1465-8. [Erratum, *Neuroreport* 1999;10(9):inside back cover.]
64. Hara H, Friedlander RM, Gagliardini V, et al. Inhibition of interleukin 1 beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *Proc Natl Acad Sci U S A* 1997;94:2007-12.
65. Kang SJ, Wang S, Hara H, et al. Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J Cell Biol* 2000;149:613-22.
66. Benchaou A, Guegan C, Courtaud C, et al. Specific caspase pathways are activated in the two stages of cerebral infarction. *J Neurosci* 2001;21:7127-34.
67. Zhu S, Stavrovskaya IG, Drozda M, et al. Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature* 2002;417:74-8.
68. Martinou JC, Dubois-Dauphin M, Staple JK, et al. Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* 1994;13:1017-30.
69. Plesnila N, Zinkel S, Le DA, et al. BID mediates neuronal cell death after oxygen-glucose deprivation and focal cerebral ischemia. *Proc Natl Acad Sci U S A* 2001;98:15318-23.
70. Schielke GP, Yang GY, Shivers BD, Betz AL. Reduced ischemic brain injury in interleukin-1 beta converting enzyme-deficient mice. *J Cereb Blood Flow Metab* 1998;18:180-5.
71. Fink K, Zhu J, Namura S, et al. Prolonged therapeutic window for ischemic brain damage caused by delayed caspase activation. *J Cereb Blood Flow Metab* 1998;18:1071-6.
72. Garden GA, Budd SL, Tsai E, et al. Caspase cascades in human immunodeficiency virus-associated neurodegeneration. *J Neurosci* 2002;22:4015-24.
73. Klivenyi P, Andreassen O, Ferrante RJ, Schleicher JR Jr, Friedlander RM, Beal MF. Transgenic mice expressing a dominant negative mutant interleukin-1 beta converting enzyme show resistance to MPTP neurotoxicity. *Neuroreport* 1999;10:635-8.
74. Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. *N Engl J Med* 2001;344:1688-700.
75. Rosen DR, Siddique T, Patterson D, et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* 1993;362:59-62. [Erratum, *Nature* 1993;364:362.]

76. Gurney ME, Pu H, Chiu AY, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 1994;264:1772-5. [Erratum, *Science* 1995;269:149.]

77. Klivenyi P, Ferrante RJ, Matthews RT, et al. Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nat Med* 1999;5:347-50.

78. Martin LJ. Neuronal death in amyotrophic lateral sclerosis is apoptosis: possible contribution of a programmed cell death mechanism. *J Neuropathol Exp Neurol* 1999; 58:459-71.

79. Guegan C, Vila M, Rosoklja G, Hays AP, Przedborski S. Recruitment of the mitochondrial-dependent apoptotic pathway in amyotrophic lateral sclerosis. *J Neurosci* 2001;21:6569-76.

80. Kostic V, Jackson-Lewis V, de Bilbao F, Dubois-Dauphin M, Przedborski S. Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science* 1997;277:559-62.

81. Harper PS. Huntington's disease. London: W.B. Saunders, 1991.

82. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 1993;72:971-83.

83. Mangiarini L, Sathasivam K, Seller M, et al. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 1996;87:493-506.

84. Reddy PH, Williams M, Charles V, et al. Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. *Nat Genet* 1998;20:198-202.

85. Ferrante RJ, Andreassen OA, Jenkins BG, et al. Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. *J Neurosci* 2000;20:4389-97.

86. Li SH, Lam S, Cheng AL, Li XJ. Intracellular huntingtin increases the expression of caspase-1 and induces apoptosis. *Hum Mol Genet* 2000;9:2859-67.

87. Kiechle T, Dedeoglu A, Kubilus J, et al. Cytochrome c and caspase-9 expression in Huntington's disease. *Neuromolecular Med* 2002;1:183-95.

88. Sanchez I, Xu CJ, Jiao P, Kakizaka A, Bleenis J, Yuan J. Caspase-8 is required for cell death induced by expanded polyglutamine repeats. *Neuron* 1999;22:623-33.

89. Zuccato C, Ciampolla A, Rigamonti D, et al. Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science* 2001;293:493-8.

90. Rigamonti D, Bauer JH, De-Fraja C, et al. Wild-type huntingtin protects from apoptosis upstream of caspase-3. *J Neurosci* 2000;20:3705-13.

91. Rigamonti D, Sipione S, Goffredo D, Zuccato C, Fossale E, Cattaneo E. Huntingtin's neuroprotective activity occurs via inhibition of procaspase-9 processing. *J Biol Chem* 2001;276:14545-8.

92. Goldberg YP, Nicholson DW, Rasper DM, et al. Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat Genet* 1996;13:442-9.

93. Wellington CL, Ellerby LM, Hackam AS, et al. Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J Biol Chem* 1998;273: 9158-67.

94. Nucifora FC Jr, Sasaki M, Peters MF, et al. Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. *Science* 2001;291:2423-8.

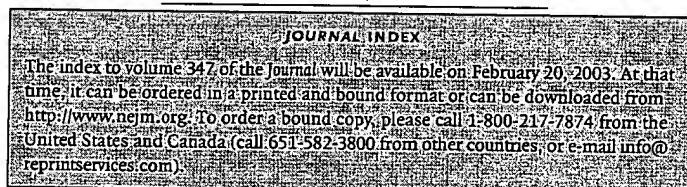
95. Cha JH, Kosinski CM, Kerner JA, et al. Altered brain neurotransmitter receptors in transgenic mice expressing a portion of an abnormal human Huntington disease gene. *Proc Natl Acad Sci U S A* 1998;95:6480-5.

96. Tikka TM, Koistinaho JE. Minocycline provides neuroprotection against N-methyl-D-aspartate neurotoxicity by inhibiting microglia. *J Immunol* 2001;166:7527-33.

97. Sanchez Mejia RO, Ona VO, Li M, Friedlander RM. Minocycline reduces traumatic brain injury-mediated caspase-1 activation, tissue damage, and neurological dysfunction. *Neurosurgery* 2001;48:1393-401.

98. Shuman SL, Bresnahan JC, Beattie MS. Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. *J Neurosci Res* 1997;50:798-808.

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Review

Neuroprotection and neurodegenerative disease

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Summary This paper will focus on commonalities in the aetiology and pathology in five areas of neurological disease with illustrative examples of therapy. Possibilities of multimodal and neuroprotective therapies in human disease, employing currently available drugs and showing evidence of neuroprotective potential in animal models, are discussed. By definition, neuroprotection is an effect that may result in salvage, recovery or regeneration of the nervous system, its cells, structure and function.¹ It is thought that there are many neurochemical modulators of nervous system damage. In epilepsy, excessive glutamate-mediated neurotransmission, impaired voltage sensitive sodium and calcium channel functioning, impaired GABA-mediated inhibition and alterations in acid base balance, when set in motion, may trigger a cascade of events leading to neuronal damage and cell death. Acute and chronic nervous system damage in response to an insult may lead to acute or delayed neuronal death, apoptotic cell death, neuronal degeneration, injury and loss, and gliosis. Cell death in the CNS following injury can occur in the manner of apoptosis, necrosis or hybrid forms.² In general, NMDA receptor and non-NMDA receptor mediated excitotoxic injury results in *neurodegeneration* along an apoptosis-necrosis continuum. The effects of neuronal injury depend on factors including the degree of brain maturity or site of the lesion. There is some evidence supporting the hypothesis that neuroprotection may be a practical and achievable target using drugs already available, at present employed only for limited indications. Using these drugs early in the disease, may save decades of development of new drugs, which would require evaluation in animal studies, and human clinical trials. New drugs would also need to be shown to be safe and acceptable, physiologically not detrimental to humans and free from idiosyncratic adverse effects. © 2002 Harcourt Publishers Ltd

NEURODEGENERATION AND THE SITE OF LESION

Neurodegenerative diseases are characterised by progressive dysfunction and death of neurons. Disorders with specific known causes are, by convention, excluded. The main degenerative disorders manifest predominantly as movement disorders, those of cognition or a mixture of both. Movement disorders include akinetic and rigid forms, predominantly extrapyramidal deficits, hyperkinetic disregulation of movement including degeneration involving the basal ganglia, ataxic with features of cerebellar ataxia, and motor neuron disorders.³ Dementia includes temporal and parietal lobe degenerations with memory disturbance and parietal lobe dysfunction, frontotemporal with apathy, disinhibition, depression and memory disturbance. Multifocal degenerations feature variable cortical and subcortical deficits. The classification is syndromic, with features being related to the site of neurons in the CNS. This classification gives no aetiological insight.⁴

AETIOLOGICAL FACTORS

Speculating on commonalities of aetiology and pathology in neurodegenerative diseases, the underlying theme may be perceived that although the clinical manifestations are quite varied, this is partly due to the fact that individual sections of the CNS are geared to perform different functions (e.g. extrapyramidal system defects cause movement disorders, but insults to cortical areas lead to cognitive impairment or seizure disorders).⁵ If the processes of neurodegeneration are augmented by comparable excitotoxicity, i.e. genetic influences interact with external

insults in various disease forms, then similar modalities of treatment may be able to conceivably address essentially similar common pathological processes and thus prevent the neuron reaching the final common pathway, which may involve mitochondrial dysfunction.⁶

Environmental factors in neurodegenerative disease comprise physical, toxic and infection related factors. Trauma is one possibility and may be epidemiologically related to Alzheimer's Disease (AD) and Motor Neuron Disease (MND). Toxic causes (e.g. metals) may be related, but none shown to be causative. Dietary excitotoxic amino acids may be implicated in endemic forms of MND but none are shown to be causative. Bacterial infections may lead to direct immune-mediated responses to unusual infections. Although several viral agents are linked to AD and MND, none causatively.³

EXCITOTOXICITY

Excitotoxicity, cerebral ischaemia and target deprivation result in varying contributions to cell damage. Degeneration may be mediated by causal mechanisms which may overlap temporarily.⁷ Olney in the 1980s proposed that increased glutamate binding to postsynaptic receptors causes channels to open with sodium and calcium entering the postsynaptic cell, causing depolarisation which initiates a cascade that leads to neuronal death.⁸ A comparison with other conditions of cerebral energy deprivation in cases of hypoxia or hypoglycaemia suggest a role for excitotoxicity, initiated by excitatory amino acid neurotransmitters. Additional factors may be peroxynitrite and oxygen free radicals.^{2,9,10}

A chain of events leading to cell damage may comprise an initiating factor, e.g. excitotoxicity, oxidative stress, growth factor withdrawal, cytokines or toxins. This may lead to apoptotic DNA fragmentation, cellular fragmentation and engulfment of the cell.³ Damage may be inflicted by excessive stimulation of glutamate receptors. A neuron expresses multiple types of glutamate receptors which may be ionotropic or metabotropic. A metabolic imbalance in the neuron leads to

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expression of immediate early genes such as c-fos, c-jun, jun-a, or jun-b. Cytoskeletal and membrane damage initially occurs in neuronal dendrites which swell and this is followed by neuronal death, but in some systems this has been shown to occur by apoptosis which is an active process of cell destruction and shrinkage, chromatin aggregation, extensive genomic fragmentation and nuclear pyknosis.

Necrosis is a common feature in many conditions, and together with apoptosis may contribute to neuronal death.⁵ Necrosis is characterised by cell swelling, rapid energy loss, general disruption of ionic and internal homeostasis, membrane lysis, release of intracellular constituents, local inflammatory reactive oedema and injury to surrounding tissue.

Indirect excitotoxicity occurs as a result of interruption of chemical synthesis performed by aerobic generation of energy. There is an altered energy production in mitochondria, a fall in membrane potential, opening of the NMDA receptor subtype, further ion entry, increased calcium load and necrotic or apoptotic cell death.¹¹ Neurotoxicity thus initiated and mediated by glutamate is thought to play a role in cerebral ischaemia, progressive neurodegenerative disease and AD.¹²

MITOCHONDRIAL FUNCTION

Beal's (1996) suggestion for a central role for defective mitochondrial energy production resulting in increased levels of free radicals, is gaining recognition. Defects in energy may contribute to excitotoxicity and oxidative damage.¹³ Evidence implicating energy defects in neurodegenerative disease comes from similarities to known mitochondrial disorders. The majority of patients with mitochondrial disease have neuronal loss, gliosis and degeneration. There is an overlap but the nature and type of CNS lesions often allows a specific diagnosis. A number of different defects of cerebral energy metabolism are associated with common patterns in neuropathology. It is suggested that chronic activation of NMDA receptors by glutamate is toxic to cultured neurons. There is excessive calcium entry which is accumulated by intracellular mitochondria, and affects mitochondrial membrane potential and ATP synthesis, glycolysis, and reactive oxygen species generation, resulting in failure of homeostasis and cell death.¹⁴ Simpson and Russell indicate that mitochondrial calcium uptake and release has important consequences for neuronal and glial activity. Neuronal death due to NMDA involved calcium entry can be delayed by inhibitors of mitochondrial permeability transition pores. Thus, mitochondrial dysfunction is increasingly implicated in neurodegenerative conditions.¹⁵

It appears there may be a commonality in aetiological factors and an important role for glutamate-mediated excitotoxicity, which denotes a final common pathway. The process could conceivably be modulated by neuroprotective agents, affecting calcium channel function, not necessarily at the neuronal membrane, but rather at the mitochondrial transition pore level.

1. EPILEPSY AND DRUGS FOR ITS TREATMENT

Many seizure disorders, particularly of generalised epilepsy, are under polygenic control. It is difficult to ascertain the exact contribution of all the mutations present. Some rare partial epilepsy syndromes have also been identified, which are genetically determined.¹⁶ Environmental factors such as trauma, infections and fever also play an aetiological role.

In developing therapeutic strategies, the question may be asked "How do we bridge the gap between the improvement in genetic technology and understanding of mutations on the one

hand, and pharmacological approaches on the other, and can any of the antiepileptic agents have a role beyond the symptomatic control of seizures, i.e. by halting the epileptic process and having a truly antiepileptogenic or neuroprotective effect?" Whilst animal models will elucidate this, human clinical trial outcome measurements such as seizure freedom, lack of status epilepticus and lack of cognitive decline will provide definitive proof of a truly neuroprotective effect.

Postulated mechanisms for antiepileptic drug (AED) actions comprise those on ion channels, GABA and glutamate systems and second messengers. At a cellular level, mitochondrial mechanisms may have a role in stabilising neurons and protecting them from insults resulting from excess calcium influx.

Drugs with multiple sites of action: Topiramate (TPM) is a relatively safe drug approved for use in epilepsy. Its profile is favourable; it has multiple mechanisms of action and a potential to exert neuroprotective effects.^{17,18} Acquisition of kindled hippocampal seizures in rats exposed to TPM indicates that control animals become kindled earlier and acquire more marked degrees of seizure severity compared to those treated with TPM, which dose dependently delays the occurrence of kindled seizures and reduces their severity.¹⁹ TPM prevents perinatal hypoxia in animal models, and enhances nerve regeneration. Yang and colleagues reported a study in rats, where infarct volume of the brain was demonstrably reduced after arterial embolisation following TPM treatment.²⁰ Its hypothetical neuroprotective effect is explained by an effect on the non-NMDA, kainate/AMPA receptor and an influence on calcium channels and sodium channels, which is important in the ischaemic model. TPM potentiates GABA activity at the GABA-receptor site. There may be an interaction between TPM and benzodiazepines at a novel, non-benzodiazepine site. Other AMPA/kainate receptor antagonists also inhibit the damage caused by perinatal hypoxia. The favourable effect produced by TPM on hippocampal kindling is not seen with traditional AEDs.

Other AEDs also have multiple mechanisms of action. Clonazepam has a specific action on the mitochondrial membrane.²¹ Valproate has either directly, or via active metabolites, a calcium channel blocking action, shown by efficacy in absence seizures.²² Lamotrigine is likely to have some neuroprotective effect, by inhibiting neurotoxicity induced by kainic acid, perhaps by an indirect rather than a direct effect on glutamate release.^{23,24} Their neuroprotective potential is not as yet fully explored.

2. MOTOR NEURON DISEASE (MND)

Clinically, MND consists of a progressive weakness affecting skeletal muscle groups. It is patchy in onset with linear progression in a given muscle group for about 18 months before total failure (4–6% deterioration per month). Recent research suggests a combined aetiological role for genetic factors and excitotoxic damage. Other possible aetiological factors include deficiencies of neurotrophic factors and autoantibodies to L-type calcium channels. Cell death in MND involves genetic, oxidative stress, glutamate related toxicity and damage to critical target proteins in organelles. The oxidative stress leads to an increased metabolic rate and a high mitochondrial activity. Glutamate related toxicity is associated with calcium permeability and AMPA receptors, and possible lack of calcium binding proteins.²⁵ The known genetic contributions are that 5–10% of patients with a common form of MND, Amyotrophic Lateral Sclerosis (ALS), have an autosomal dominant inherited form of disease. Mutations of the Cu/Zn superoxide dismutase (SOD1) gene on chromosome 21q account for about

25% of all familial cases. SOD1 has a role in removing superoxide radicals.²⁶ Motor neuron loss in the genetically modified animal model (transgenic mice) is significantly higher than that observed in controls. At 18 weeks there is significant spinal motor neuron loss leading to death of the animal at 39 weeks, whereas motor neuron counts in a control group remain stable (Feeney S et al., 2000, personal communication). Reactive astrocytosis, another parameter of disease progression in SOD1 mice, shows an increase in the number of astrocytes measured in spinal cord of SOD1 mice. Onset of the disease occurs at about 28 weeks, paralysis at 32 weeks and end stage at 39 weeks. Existence of an animal model and long delay between onset of measurable parameters of paralytic stage and death, allows a window of opportunity to treat the disorder by potential neuroprotective drugs. Actions of a drug riluzole are currently being tested for slowing the progression of MND. TPM has a wider range of postulated mechanisms of action compared to riluzole. Studies involving the use of neuroprotective agents in the treatment of MND are in progress.

3. PARKINSON'S DISEASE (PD)

Manifestations of PD are attributable to reduced dopaminergic input into the striatum, due to neuronal degeneration in the pars compacta of the substantia nigra. Aetiological factors are complex. PD is inherited as an autosomal dominant trait in some families. Genetically, mutations called PARK-1 (a synuclein), PARK-2 and PARK-3, 4 etc. have been well characterised in this disorder.²⁷ Environmental toxins and oxidative stress and mitochondrial dysfunction may exert a contribution to nigral degeneration.

Free radical damage and defective mitochondrial oxidative phosphorylation are more common in PD patients than controls. Free radicals are believed to cause damage to mitochondrial DNA and other macromolecules. This brings PD in line with disorders mentioned earlier as a neurodegenerative disease.²⁸ There is a similarity between PD and the disorder produced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication.²⁹

Parkinson's Disease is the commonest of the Lewy body diseases. There are many other diseases which have this characteristic structure noted on histology. The formation of Lewy bodies may be cytoprotective; viz., a means of removing damaged cytoskeletal proteins. Constituent proteins in the Lewy bodies can be divided into functional proteins and incorporated proteins, probably in the process of being degraded. These include tubulin and microtubular associated proteins, amyloid precursor protein and synaptic protein. In PD there is a gradual loss of neurons, which may provide an opportunity for halting the destructive process with the use of neuroprotective agents.

Selegiline is a potent, selective, irreversible, monoamine oxidase-B inhibitor, present as a glial enzyme in the brain, with multiple mechanisms of action. It interferes with the uptake of catecholamines and indirectly acting sympathomimetics.³⁰ Selegiline enhances superoxide dismutase and catalase activity in the striatum. It also facilitates activity of the catecholaminergic system and protects the nigrostriatal neurons against 6OH-dopamine, MPTP, DSP-4, all of which are toxic to the brain. Selegiline causes a fall in dopamine turnover, which has been regarded toxic to neurons by creating an increase in toxic radicals. In humans, a greater number of medial striatal neurons have been shown to survive in patients treated with selegiline.^{31,32} There were fewer Lewy bodies in selegiline treated patients than in controls. In rats, selegiline delays the decline in learning and memory and increases life span. In rodents,

selegiline protects substantia nigra neurons by blocking the conversion of MPTP to MTP. Rat facial motor neurons dying after axotomy due to lack of trophic support, are partly protected after selegiline treatment, indicating a trophic action. Certainly in animal models and potentially in humans, selegiline has a neuroprotective effect. However, because of a symptomatic effect on PD, the effect of neuroprotection is difficult to dissect from symptomatic effects. Several clinical trials were terminated too early to define differences between neuroprotection and symptomatic effect.³³ Speculatively, a putative neuroprotective agent like selegiline may be combined with a neuroprotective agent acting via glutamate receptors, ion channels or GABAergic effects such as exerted by TPM. A current multicentre U.S. study uses riluzole as a potential neuroprotective agent in PD. There is no plan to combine riluzole with selegiline but such combinations may need exploration.³⁴

4. MULTIPLE SCLEROSIS (MS)

MS is usually associated with demonstrable foci of demyelination using magnetic resonance imaging (MRI), and oligoclonal bands of immunoglobulins on electrophoresis of CSF. It often pursues a relapsing and remitting course, is progressive from the outset or becomes progressive after initial remissions. MS patients have significantly greater antibody levels to measles virus than controls. Compared to their unaffected monozygotic twins, MS patients show restricted expression of certain T-cell receptor genes, and patients with MS show abnormal T-cell responses to several white matter antigens including myelin-basic protein, myelin-oligodendrocyte glycoprotein and crystalline. Crystalline is expressed by oligodendroglia and astrocytes in MS plaques but not in normal white matter.³⁵ In some patients with MS, inflammatory mechanisms of demyelination are important, but in others dystrophic features are noted leading to cell death without marked inflammation.³⁶ Numerous studies of immunomodulatory therapy suggest that interferons (IFN) are beneficial whilst relapses and exacerbations occur, but are ineffective once true progression has occurred.

Until recently emphasis in MS research focused on demyelination, although there is evidence that genetic factors as expressed by neuronal susceptibility to myelin basic protein may underlie the clinical response to environmental insults, thus shifting the primary focus from the myelin related inflammatory response to a neuronal susceptibility to which demyelination is secondary. This may be influenced by neuroprotection. Axonal death or survival may be the key determinant of severity of disability in progressive disease. Neuropathologically demyelinated fibres are more susceptible to transection and death.³⁷ There are variations in immunological mechanisms between patients in different stages of MS. MRI and MRI spectroscopy have shifted interest from obvious white matter lesions to normal appearing white matter where axonal loss is demonstrable early. It is proposed that there may be a role for multiple level therapy using immunomodulatory drugs (IFN), ablative therapy with autologous haematopoietic stem cell rescue immunosuppressants (such as cytotoxic therapy and steroids) and neuroprotective agents from the beginning, attempting to protect neurons from external, inflammatory and possible later mitochondrial damage, by way of mitochondrial calcium dysregulation.^{38,39}

5. ALZHEIMER'S DISEASE (AD)

The role of genetic contribution in AD has been extensively studied.⁴⁰ The variety of chromosomes known to be involved in

types of AD include chromosome-21 manifesting from birth in Downs Syndrome, presenilin-1 associated with chromosome-14, presenilin-2 associated with chromosome-1, amyloid precursor protein associated with chromosome-21 and chromosome-19 associated with apolipoprotein-E and stability of amyloid protein deposits.

Neurotoxicity, mediated by glutamate, is thought to play a role in AD as in other progressive neurodegenerative diseases.¹² The immune system may also play a role. Induction of neurotoxic microglia by senile plaques may be a focus for immunosuppressive drugs. Similarities exist between neuronal injury and cell death in age related dementia, and focal cerebral ischaemia. After stroke, excitotoxins may cause neuronal death and also apoptosis of some neurons. HIV-1 related dementia is associated with abnormalities caused by immune related toxins.⁴¹ In AD the molecular pathology of tau protein may have an important role, although not necessarily pivotal.

Normal tau proteins are microtubular binding proteins, predominantly axonal, which stabilise the neuronal cytoskeleton. Several lines of evidence point to the primary role of A β amyloid in the pathogenesis of AD. Some mutations in the amyloid precursor protein gene have been linked with rare familial forms of AD. The neuropathological findings in these cases are indistinguishable from those of sporadic AD and include the presence of many neurofibrillary tangles (NFTs). The amyloid cascade proposes a central role for A β amyloid in the pathogenesis of AD, but the link between A β amyloid protein generation and the formation of NFTs is not known. Cognitively normal individuals can have large numbers of neocortical plaques although predominantly of the diffuse type. Multiple aetiologies are hardly mutually exclusive. Mutations in presenilin genes, which are causally linked to cases of early onset inherited AD, may increase vulnerability of cholinergic neurons to apoptosis. The mechanism may involve perturbed calcium regulation and mitochondrial dysfunction.^{42,43}

It may be possible to conceive multimodal therapy to include administration of an approved acetylcholinesterase inhibitor, designed to address the deficit of impaired acetylcholine transmission, together with antiinflammatory drugs of the NSAID type, showing a trend towards improving cognitive function, plus platelet antiaggregants to diminish vascular insults and ischaemic effects. If there were immunological impairments leading to neuronal death in AD, immunological function should be addressed.⁴⁴ Neuroprotective drugs should be also considered, which in the later phases of the disease may prevent neuronal necrosis. It is possible that a final common pathway for neuronal susceptibility may operate, similar to that observed in stroke and other neurodegenerative processes.⁴¹

In support for multimodal therapy, a link may be drawn with other diseases, such as leukaemia and Hodgkin's Disease (HD). Improvement in survival in HD has changed dramatically over the past decades from less than 50% to 94% expected to survive in the year 2000. Multi-modality therapy for HD involves intensive field radiation of a moderate area, monoclonal antibody therapy, limited cycles of multi-agent chemotherapy comprising mechlorethamine, vincristine, procarbazine and prednisolone, doxorubicin, bleomycin, vinblastine and dacarbazine. These drugs are the bases of the Beacopp regimen.⁴⁵

In summary, it is suggested that a mechanism and final common pathway of cell destruction in a variety of neurological diseases involves glutamate triggered excitotoxicity, NMDA receptor operated channels, excessive calcium influx, and generation of free radicals which leads to neuronal damage. It appears likely that this final common pathway involves existing

mitochondrial mechanisms. Five clinically important areas of neurological disease, where treatment is less than ideal were highlighted. Translational research applying striking achievements of basic scientists to clinical problems may be justified. Emerging ideas re aetiology and pathology, which may lead to improved therapeutics involving multimodality and neuroprotection, were presented.

REFERENCES

- White HS. Basic research update: Evidence for neuroprotection with Topiramate. Proc. 3rd Annual Topamax Symposium, Satellite Meeting of Epilepsy Society of Australia; Annual Scientific Meeting, Adelaide, 1999.
- Leist M and Nicotera P. Apoptosis, excitotoxicity and neuropathology. *Exp Cell Res* 1998; 239: 183–201.
- Ellison D and Love S. Neurodegenerative disorders. In: *Neuropathology, a reference text of CNS pathology*. Ellison D and Love S (eds), Harcourt Publishers, London; 2000: 26.1–33.11.
- Ellison D and Love S. Dementias. In: *Neuropathology, a reference text of CNS pathology*. Ellison D and Love S (eds), Harcourt Publishers, London; 2000: 31.1–31.34.
- Nicotera P and Lipton SA. Excitotoxins in neuronal apoptosis and necrosis. *J Cerebral Blood Flow & Metab* 1999; 19: 583–591.
- Montal M. Mitochondria, glutamate neurotoxicity and the death cascade. *Biochim et Biophys Acta* 1998; 1366: 113–126.
- Martin LJ, Al-Abdulla NA, Brambrink AM, Kirsch JR and Sieber FE. Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis. *Brain Res Bull* 1998; 46: 281–309.
- Olney JW. Glutamate induced retinal degeneration in neonatal mice. *Electron microscopy of the acutely evolving lesion*. *J Neuropathol Exp Neurol* 1969; 28: 455–474.
- Brown GK and Squier MV. Neuropathology and pathogenesis of mitochondrial diseases. *J Inher Metab Dis* 1996; 19: 553–572.
- Lipton SA, Choi YB, Sucher NJ and Chen HS. Neuroprotective versus neurodestructive effects of NO-related species. *Biofactors* 1998; 8: 33–40.
- Ludolph AC, Meyer T and Riepe MW. The role of excitotoxicity in ALS – what is the evidence? *J Neurol* 2000; 247(Suppl 1): I7–I16.
- Arias C, Becerra-Garcia F and Tapia R. Glutamic acid and Alzheimer's disease. *Neurobiology* 1998; 6: 33–43.
- Beal MF. Mitochondria, free radicals and neurodegeneration. *Curr Opin Neurobiol* 1996; 6: 661–666.
- Nichols DG and Budd SL. Neuronal excitotoxicity: the role of mitochondria. *Biofactors* 1998; 8: 287–299.
- Simpson PB and Russell JT. Role of mitochondrial Ca²⁺ regulation in neuronal and glial cell signalling. *Brain Res Rev* 1998; 26: 72–81.
- Scheffer IE, Bhatia KP, Lopes-Cendes I et al. Autosomal dominant nocturnal frontal lobe epilepsy. A distinctive clinical disorder. *Brain* 1995; 118: 61–73.
- White HS. Comparative anticonvulsant and mechanistic profile of the established and newer antiepileptic drugs. *Epilepsia* 1999; 40(Suppl 5): S2–S10.
- Perucca E. A pharmacological and clinical review on Topiramate, a new antiepileptic drug. *Pharmacol Rev* 1997; 35: 241–256.
- Hanaya R, Sasa M, Ujihara H et al. Suppression by topiramate of epileptiform burst discharges in hippocampal CA3 neurons of spontaneously epileptic rat in vitro. *Brain Res* 1998; 789: 274–282.
- Yang Y, Shuaib A, Qiu L and Siddiqui MM. Neuroprotection by delayed administration of topiramate in a rat model of middle cerebral artery embolization. *Brain Res* 1998; 804: 169–176.
- Macdonald RL. Cellular actions of antiepileptic drugs. In: *Antiepileptic drugs: Pharmacology and Therapeutics*. Eadie MJ and Vajda FJE (eds), Springer, 1999: 123–150.
- Walker MC and Sander JWAS. Lamotrigine. In: *Antiepileptic drugs: Pharmacology and Therapeutics*. Eadie MJ and Vajda FJE (eds), Springer, 1999: 331–358.
- Meldrum RS. Pharmacology and mechanism of action of lamotrigine. In: *Lamotrigine – a new advance in the treatment of epilepsy*. Reynolds EH (ed), Royal Society of Medicine Services, London; 1993: 5–10.
- Shaw PJ and Ince PG. Glutamate, excitotoxicity and amyotrophic lateral sclerosis. *J Neurol* 1997; 244(Suppl 2): S3–S14.
- Robberecht W. Oxidative stress in amyotrophic lateral sclerosis. *J Neurol* 2000; 247 (Suppl 1): I1–I6.

26. Ellison and Love S. Motor Neuron Disorders. In: *Neuropathology, a reference text of CNS pathology*. Ellison D and Love S (eds), Harcourt Publishers, London; 2000: 27.1-27.10.
27. Ellison D and Love S. Parkinson's Disease. In: *Neuropathology, a reference text of CNS pathology*. Ellison D and Love S (eds), Harcourt Publishers, London; 2000: 28.1-28.16.
28. Jenner P and Olanow CWT. Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology* 1996; 47(6 Suppl 3): S161-S170.
29. Langston JW. The aetiology of Parkinson's disease with emphasis on the MPTP story. *Neurology* 1996; 47(6 Suppl 3): S153-S160.
30. Gerlach M, Youdim MBH and Reiderer P. Pharmacology of selegiline. *Neurology* 1996; 47(6 Suppl 3): S137-S145.
31. Tatton WG, Chalmers-Redman RM. Modulation of gene expression rather than monoamine oxidase inhibition: (-)-deprenyl-related compounds in controlling neurodegeneration. *Neurology* 1996; 47(6 Suppl 3): S171-S183.
32. Rinne UK, Rinne JO, Riene JK, Lakso K and Lonnberg P. Brain neurotransmitters and neuropeptides in Parkinson's disease. *Acta Physiol Pharmacol Latinoam* 1984; 34: 287-299.
33. Olanow CW. Selegiline: current perspectives on issues related to neuroprotection and mortality. *Neurology* 1996; 47(6 Suppl 3): S210-S216.
34. Burkhardt K, Schwarz J and Storch A. Ribuzole protects dopamine neurons against oxidative stress and impairment of cellular energy supply. *Neurology* 2000; 54(Suppl 3): A52.
35. Ellison D and Love S. Multiple Sclerosis. In: *Neuropathology, a reference text of CNS pathology*. Ellison D and Love S (eds), Harcourt Publishers, London; 2000: 19.1-19.12.
36. Lassmann H. Neuropathology in multiple sclerosis: new concepts. *Multiple Sclerosis* 1998; 4: 93-98.
37. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S and Bo L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med* 1998; 338: 278-285.
38. Cohen BA, Karlin KH, Lobeck L, et al. Intense immune ablative therapy with autologous hematopoietic stem cell rescue for progressive multiple sclerosis: a pilot safety study. *Neurology* 2000; 54(Suppl 3): A59.
39. Whartenby KA, Mills PC, Rogg J, Wilterdink J and Calabresi PA. An open label trial of combination MS therapy using IFN β 1a and oral methotrexate 20 mg weekly. *Neurology* 2000; 54(Suppl 3): A24.
40. Ellison D and Love S. Alzheimer's Disease. In: *Neuropathology, a reference text of CNS pathology*. Ellison D and Love S (eds), Harcourt Publishers, London; 2000: 31.1-31.34.
41. Lipton SA. Similarity of neuronal cell injury and death in AIDS dementia and focal cerebral ischaemia: potential treatment with NMDA open-channel blockers and nitric oxide-related species. *Brain Pathol* 1996; 6: 507-517.
42. Mattson MP and Pedersen WA. Effects of amyloid precursor protein derivatives and oxidative stress on basal forebrain cholinergic systems in Alzheimer's disease. *Int J Dev Neurosci* 1998; 16: 737-753.
43. Blass J and Poirier J. Pathophysiology of the Alzheimer syndrome. In: *Clinical diagnosis and management of Alzheimer's Disease*. Gauthier S (ed), Martin Dunitz, London; 1996: 17-31.
44. Giulian D. A strategy for identifying immunosuppressive therapies for Alzheimer Disease. *Alzheimer Disease & Associated Disorders* 1998; 12(Suppl 2): S7-S14.
45. Engert A, Wolf J and Diehl V. Treatment of advanced Hodgkin's lymphoma: standard and experimental approaches. *Semin Hematol* 1999; 36: 282-289.



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<p>(54) Title: ACTIVATED T CELLS, NERVOUS SYSTEM-SPECIFIC ANTIGENS AND THEIR USES</p> <p>(57) Abstract</p> <p>Compositions and methods are provided for treating injury to or disease of the central or peripheral nervous system. In one embodiment, treatment is effected using activated T cells that recognize an antigen of the nervous system or a peptide derived therefrom or a derivative thereof to promote nerve regeneration or to prevent or inhibit neuronal degeneration within the nervous system. Treatment involves administering an NS-specific antigen or peptide derived therefrom or a derivative thereof, or a nucleotide sequence encoding said antigen or peptide, to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the nervous system, either the central nervous system or the peripheral nervous system. The NS-specific activated T cells can be administered alone or in combination with NS-specific antigen or peptide derived therefrom or a derivative thereof or a nucleotide sequence encoding said antigen or peptide, or any combination thereof.</p>																			
<table border="1"> <caption>Data from Bar Chart: T cell numbers/mm²</caption> <thead> <tr> <th>Group</th> <th>T cell numbers/mm²</th> </tr> </thead> <tbody> <tr> <td>T45</td> <td>~250</td> </tr> <tr> <td>Tp277</td> <td>~180</td> </tr> <tr> <td>Tov</td> <td>~170</td> </tr> <tr> <td>PBS</td> <td>~50</td> </tr> <tr> <td>Tp277</td> <td>~60</td> </tr> <tr> <td>Tov</td> <td>~50</td> </tr> <tr> <td>PBS</td> <td>~50</td> </tr> </tbody> </table>				Group	T cell numbers/mm²	T45	~250	Tp277	~180	Tov	~170	PBS	~50	Tp277	~60	Tov	~50	PBS	~50
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**ACTIVATED T CELLS, NERVOUS SYSTEM-SPECIFIC ANTIGENS
AND THEIR USES**

Field of the Invention

The present invention relates to compositions and methods for the promotion of nerve regeneration or prevention or inhibition of neuronal degeneration to ameliorate the effects of injury or disease of the nervous system (NS). In certain embodiments, activated antiself T cells, an NS-specific antigen or peptide derived therefrom or a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom can be used to promote nerve regeneration or to prevent or inhibit neuronal degeneration caused by injury or disease of nerves within the central nervous system or peripheral nervous system of a human subject. The compositions of the present invention may be administered alone or may be optionally administered in any desired combination.

Background of the Invention

The nervous system comprises the central (CNS) and the peripheral (PNS) nervous system. The central nervous system is composed of the brain and spinal cord; the peripheral nervous system consists of all of the other neural elements, namely the nerves and ganglia outside of the brain and spinal cord.

Damage to the nervous system may result from a traumatic injury, such as penetrating trauma or blunt trauma, or a disease or disorder, including but not limited to Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis (ALS), diabetic neuropathy, senile dementia, and ischemia.

Maintenance of central nervous system integrity is a complex "balancing act" in which compromises are struck with the immune system. In most tissues, the immune system plays an essential part in protection, repair, and healing. In the central nervous system, because of its unique immune privilege, immunological reactions are relatively limited (Streilein, J.W., 1993, *Curr. Opin. Immunol.* 5:428-423; Streilein, J.W., *Science* 270:1158-1159). A growing body of evidence indicates

that the failure of the mammalian central nervous system to achieve functional recovery after injury is a reflection of an ineffective dialog between the damaged tissue and the immune system. For example, the restricted communication between the central nervous system and blood-borne macrophages affects the capacity of axotomized axons to regrow; transplants of activated macrophages can promote central nervous system regrowth (Lazarov Spiegler, O., et al, 1996, FASEB J. 19:1296-1302; Rapalino, O. et al., 1998, Nature Med. 4:814-821).

Activated T cells have been shown to enter the central nervous system parenchyma, irrespective of their antigen specificity, but only T cells capable of reacting with a central nervous system antigen seem to persist there (Hickey, W.F. et al., 1991, J. Neurosci. Res. 28:254-260; Werkele, H., 1993, In The Blood-Brain Barrier, Pardridge, Ed., Raven Press, Ltd. New York, 67-85; Kramer, R. et al., 1995, Nature Med. 1(11):1162-1166)). T cells reactive to antigens of central nervous system white matter, such as myelin basic protein (MBP), can induce the paralytic disease experimental autoimmune encephalomyelitis (EAE) within several days of their inoculation into naive recipient rats (Ben Nun, A., et al., 1981, Eur. J. Immunol. 11:195-199). Anti-MPB T cells may also be involved in the human disease multiple sclerosis (Ota, K. et al., 1990 Nature 346:183-187; Martin, R. 1997, J. Neural Transm. Suppl. 49:53-67). However, despite their pathogenic potential, anti-MBP T cell clones are present in the immune systems of healthy subjects (Burns, J., et al. 1983, Cell Immunol. 81:435-440; Pette, M. et al., 1990, Proc. Natl. Acad. Sci. USA 87:7968-7972; Martin, R. et al., 1990, J. Immunol. 145:540-548; Schiuesener, H.J., et al., 1985, J. Immunol. 135:3128-3133). Activated T cells, which normally patrol the intact central nervous system, transiently accumulate at sites of central nervous system white matter lesions (Hirschberg, D.L., et al., 1998, J. Neuroimmunol. 89:88-96).

A catastrophic consequence of central nervous system injury is that the primary damage is often compounded by the gradual secondary loss of adjacent neurons that apparently were undamaged, or only marginally damaged, by the initial injury

(Faden, A. I., et al., 1992, Trends Pharmacol. Sci. 13:29-35; Faden, A.I., 1993, Crit. Rev. Neurobiol. 7:175-186; McIntosh, T.K., 1993, J. Neurotrauma 10:215-261). The primary lesion causes changes in extracellular ion concentrations, elevation of amounts of free radicals, release of neurotransmitters, depletion of growth factors, and local inflammation. These changes trigger a cascade of destructive events in the adjacent neurons that initially escaped the primary injury (Lynch, D.R. et al., 1994, Curr. Opin. Neurol. 7:510-516; Bazan, N.G. et al., 1995, J. Neurotrauma 12:791-814; Wu, D. et al., 1994, J. Neurochem. 62:37-44). This secondary damage is mediated by activation of voltage-dependent or agonist-gated channels, ion leaks, activation of calcium-dependent enzymes such as proteases, lipases and nucleases, mitochondrial dysfunction and energy depletion, culminating in neuronal cell death (Yoshina, A. et al., 1991 Brain Res. 561:106-119; Hovda, D.A. et al., 1991, Brain Res. 567:1-10; Zivin, J.A., et al, 1991 Sci. Am. 265:56-63; Yoles, E. et al., 1992, Invest. Ophthalmol. Vis. Sci. 33:3586-3591). The widespread loss of neurons beyond the loss caused directly by the primary injury has been called "secondary degeneration."

Another tragic consequence of central nervous system injury is that neurons in the mammalian central nervous system do not undergo spontaneous regeneration following an injury. Thus, a central nervous system injury causes permanent impairment of motor and sensory functions.

Spinal cord lesions, regardless of the severity of the injury, initially result in a complete functional paralysis known as spinal shock. Some spontaneous recovery from spinal shock may be observed, starting a few days after the injury and tapering off within three to four weeks. The less severe the insult, the better the functional outcome. The extent of recovery is a function of the amount of undamaged tissue minus the loss due to secondary degeneration. Recovery from injury would be improved by neuroprotective treatment that could reduce secondary degeneration.

Citation or identification of any reference in this section or any other part of this application shall not be

construed as an admission that such reference is available as prior art to the invention.

SUMMARY OF THE INVENTION

The present invention is directed to methods and compositions for the promotion of nerve regeneration or prevention or inhibition of neuronal degeneration to ameliorate the effects of injury to or disease of the nervous system (NS). The present invention is based in part on the applicants' unexpected discovery that activated T cells that recognize an antigen of the NS of the patient promote nerve regeneration or confer neuroprotection. As used herein, "neuroprotection" refers to the prevention or inhibition of degenerative effects of injury or disease in the NS. Until recently, it was thought that the immune system excluded immune cells from participating in nervous system repair. It was quite surprising to discover that NS-specific activated T cells can be used to promote nerve regeneration or to protect nervous system tissue from secondary degeneration which may follow damage caused by injury or disease of the CNS or PNS.

"Activated T cell" as used herein includes (i) T cells that have been activated by exposure to a cognate antigen or peptide derived therefrom or derivative thereof and (ii) progeny of such activated T cells. As used herein, a cognate antigen is an antigen that is specifically recognized by the T cell antigen receptor of a T cell that has been previously exposed to the antigen. Alternatively, the T cell which has been previously exposed to the antigen may be activated by a mitogen, such as phytohemagglutinin (PHA) or concanavalin A.

In one embodiment, the present invention provides pharmaceutical compositions comprising a therapeutically effective amount of NS-specific activated T cells and methods for using such compositions to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the CNS or PNS, in an amount which is effective to ameliorate the effects of an injury or disease of the NS. "NS-specific activated T cell" as used herein refers to an activated T cell having specificity for an antigen of the NS of a patient. The antigen used to confer the specificity to the T cells may be a self NS-antigen

of the patient, a peptide derived therefrom, or an NS-antigen of another individual or even another species, or a peptide derived therefrom, as long as the activated T cell recognizes an antigen in the NS of the patient.

The NS-specific activated T cells are used to promote nerve regeneration or to prevent or inhibit the effects of disease. If the disease being treated is an autoimmune disease, in which the autoimmune antigen is an NS antigen, the T cells which are used in accordance with the present invention for the treatment of neural damage or degeneration caused by such disease are preferably not activated against the same autoimmune antigen involved in the disease. While the prior art has described methods of treating autoimmune diseases by administering activated T cells to create a tolerance to the autoimmune antigen, the T cells of the present invention are not administered in such a way as to create tolerance, but are administered in such a way as to create accumulation of the T cells at the site of injury or disease so as to facilitate neural regeneration or to inhibit neural degeneration.

The prior art also discloses uses of immunotherapy against tumors, including brain tumors, by administering T cells specific to an NS antigen in the tumor so that such T cells may induce an immune system attack against the tumors. The present invention is not intended to comprehend such prior art techniques. However, the present invention is intended to comprehend the inhibition of neural degeneration or the enhancement of neural regeneration in patients with brain tumors by means other than the prior art immunotherapy of brain tumors. Thus, for example, NS-specific activated T cells, which are activated to an NS antigen of the patient other than an antigen which is involved in the tumor, would be expected to be useful for the purpose of the present invention and would not have been suggested by known immunotherapy techniques.

The present invention also provides pharmaceutical compositions comprising a therapeutically effective amount of an NS-specific antigen or peptide derived therefrom or derivative thereof and methods of use of such compositions to promote nerve regeneration or to prevent or inhibit neuronal degeneration in the CNS or PNS, in which the amount is

effective to activate T cells *in vivo* or *in vitro*, wherein the activated T cells inhibit or ameliorate the effects of an injury or disease of the NS. "NS-specific antigen" as used herein refers to an antigen that specifically activates T cells such that following activation the activated T cells accumulate at a site of injury or disease in the NS of the patient. In one embodiment, the peptide derived from an NS-specific antigen is a "cryptic epitope" of the antigen. A cryptic epitope activates specific T cells after an animal is immunized with the particular peptide, but not with the whole antigen. In another embodiment, the peptide derived from an NS-specific antigen is an immunogenic epitope of the antigen.

"Derivatives" of NS-specific antigens or peptides derived therefrom as used herein refers to analogs or chemical derivatives of such antigens or peptides as described below, see Section 5.2.

The present invention also provides pharmaceutical compositions comprising a therapeutically effective amount of a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom or derivative thereof and methods of use of such compositions to promote nerve regeneration or for preventing or inhibiting neuronal degeneration in the CNS or PNS in which the amount is effective to ameliorate the effects of an injury or disease of the NS.

In the practice of the invention, therapy for amelioration of effects of injury or disease comprising administration of NS-specific activated T cells may optionally be in combination with an NS-specific antigen or peptide derived therefrom.

Additionally, oral administration of NS-specific antigen or a peptide derived therefrom, can be combined with active immunization to build up a critical T cell response immediately after injury.

In another embodiment cell, banks can be established to store NS sensitized T cells for neuroprotective treatment of individuals at a later time, as needed. In this case, autologous T cells may be obtained from an individual. Alternatively, allogeneic or semi-allogeneic T cells may be stored such that a bank of T cells of each of the most common

MHC-class II types are present. In case an individual is to be treated for an injury, preferably autologous stored T cells are used, but, if autologous T cells are not available, then cells should be used which share an MHC type II molecule with the patient, and these would be expected to be operable in that individual. The cells are preferably stored in an activated state after exposure to an NS antigen or peptide derived therefrom. However, the cells may also be stored in a resting state and activated once they are thawed and prepared for use. The cell lines of the bank are preferably cryopreserved. The cell lines are prepared in any way which is well known in the art. Once the cells are thawed, they are preferably cultured prior to injection in order to eliminate non-viable cells. During this culturing, the cells can be activated or reactivated using the same NS antigen or peptide as used in the original activation. Alternatively, activation may be achieved by culturing in the presence of a mitogen, such as phytohemagglutinin (PHA) or concanavalin A (preferably the former). This will place the cells into an even higher state of activation. The few days that it takes to culture the cells should not be detrimental to the patient as the treatment in accordance with the present invention may occur any time up to a week or more after the injury in order to still be effective. Alternatively, if time is of the essence, the stored cells may be administered immediately after thawing.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a bar graph showing the presence of T cells in uninjured optic nerve or in injured optic nerve one week after injury. Adult Lewis rats were injected with activated T cells of the anti-MBP (T_{MBP}), anti-OVA (T_{OVA}), anti-p277 (T_{p277}) lines, or with PBS, immediately after unilateral crush injury of the optic nerve. Seven days later, both the injured and uninjured optic nerves were removed, cryosectioned and analyzed immunohistochemically for the presence of immunolabeled T cells. T cells were counted at the site of injury and at randomly selected areas in the uninjured optic nerves. The histogram shows the mean number of T cells per $\text{mm}^2 \pm \text{s.e.m.}$, counted in two to three sections of each nerve. Each

group contained three to four rats. The number of T cells was considerably higher in injured nerves of rats injected with anti-MBP, anti-OVA or anti-p277 T cells; statistical analysis (one-way ANOVA) showed significant differences between T cell numbers in injured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells and the T cell numbers in injured optic nerves of rats injected with PBS ($P<0.001$); and between injured optic nerves and uninjured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells ($P<0.001$).

Fig. 2 is a bar graph illustrating that T cells specific to MBP, but not of OVA or p277 or hsp60, protect neurons from secondary degeneration. Immediately after optic nerve injury, rats were injected with anti-MBP, anti-OVA or anti-p277 T cells, or with PBS. The neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the site of the injury, immediately after injury (for assessment of primary damage) or two weeks later (for assessment of secondary degeneration). Five days after dye application, the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from three to five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy. RGC survival in each group of injured nerves was expressed as the percentage of the total number of neurons spared after the primary injury (42% of neurons remained undamaged after the primary injury). The neuroprotective effect of anti-MBP T cells compared with that of PBS was significant ($P<0.001$, one-way ANOVA). Anti-OVA T cells or anti-p277 T cells did not differ significantly from PBS in their effects on the protection of neurons that had escaped primary injury ($P>0.05$, one-way ANOVA). The results are a summary of five experiments. Each group contained five to ten rats.

Figs. 3 (A-C) present photomicrographs of retrogradely labeled retinas of injured optic nerves of rats. Immediately after unilateral crush injury of their optic nerves, rats were injected with PBS (Fig. 3A) or with activated anti-p277 T cells (Fig. 3B) or activated anti-MBP T cells (Fig. 3C). Two weeks later, the neurotracer dye 4-Di-10-Asp was

applied to the optic nerves, distal to the site of injury. After 5 days, the retinas were excised and flat-mounted. Labeled (surviving) RCGs, located at approximately the same distance from the optic disk in each retina, were photographed.

Figs. 4(A-B) are graphs showing that clinical severity of EAE is not influenced by an optic nerve crush injury. For the results presented in Fig. 4A, Lewis rats, either uninjured (dash line) or immediately after optic nerve crush injury (solid line), were injected with activated anti-MBP T cells. EAE was evaluated according to a neurological paralysis scale. [Data points represent \pm s.e.m.] These results represent a summary of three experiments. Each group contained five to nine rats. Fig. 4B shows that the number of RGCs in the uninjured optic nerve is not influenced by injection of anti-MBP T cells. Two weeks after the injection of anti-MBP T cells or PBS, 4-Di-10Asp was applied to the optic nerves. After 5 days the retinas were excised and flat-mounted. Labeled RGCs from five fields (located at approximately the same distance from the optic disk) in each retina were counted and the average number per mm^2 was calculated. There was no difference between the numbers of labeled RGCs in rats injected with anti-MBP T cells (T_{MBP}) and in PBS-injected control rats.

Fig. 5 is a bar graph showing that T cells specific to p51-70 of MBP protect neurons from secondary degeneration. Immediately after optic nerve injury, rats were injected with anti-MBP T cells, anti-p51-70 T cells, or PBS. The neurotracer dye 4-Di-10-Asp was applied to optic nerves distal to the site of the injury, immediately after injury (for assessment of primary damage) or two weeks later (for assessment of secondary degeneration). Five days after dye application, the retinas were excised and flat-mounted. Labeled retinal ganglion cells (RGCs) from three to five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy. RGC survival in each group of injured nerves was expressed as the percentage of the total number of neurons spared after primary injury. Compared with

that of PBS treatment, the neuroprotective effects of anti-MBP anti-p51-70 T cells were significant ($P<0.001$, one-way ANOVA).

Figs. 6(A-B) are graphs showing that anti-MBP T cells increase the compound action potential (CAP) amplitudes of injured optic nerves. Immediately after optic nerve injury, rats were injected with either PBS or activated anti-MBP T cells (T_{MBP}). Two weeks later, the CAPs of injured (Fig. 6A) and uninjured (Fig. 6B) nerves were recorded. There were no significant differences in mean CAP amplitudes between uninjured nerves obtained from PBS-injected and T cell-injected rats ($n=8$; $p=0.8$, Student's t-test). The neuroprotective effect of anti-MBP T cells (relative to PBS) on the injured nerve on day 14 after injury was significant ($n=8$, $p=0.009$, Student's t-test).

Figs. 7(A-B) are graphs showing recovery of voluntary motor activity as a function of time after contusion, with and without injection of autoimmune anti-MBP T cells. (7A) Twelve rats were deeply anesthetized and laminectomized, and then subjected to a contusion insult produced by a 10 gram weight dropped from a height of 50 mm. Six of the rats, selected at random, were then inoculated i.p. with 10^7 anti-MBP T cells and the other six were inoculated with PBS. At the indicated time points, locomotor behavior in an open field was scored by observers blinded to the treatment received by the rats. Results are expressed as the mean values for each group. The vertical bars indicate SEM. Differences tested by repeated ANOVA, including all time points, were significant ($p<0.05$). (7B) A similar experiment using five PBS-treated animals and six animals treated with anti-MBP T cells were all subjected to a more severe contusion. At the indicated time points, locomotor behavior in an open field was scored. The results are expressed as the mean values for each group. The vertical bars indicate S.E.M. Rats in the treated group are represented by open circles and rats in the control group are represented by black circles. Horizontal bars show the median values. The inset shows the median plateau values of the two groups.

Figs 8(A-C) show retrograde labeling of cell bodies at the red nucleus in rats treated with autoimmune anti-MBP T cells (8A) and in control injured (8B) rats. Three months

after contusion and treatment with anti-MBP T cells, some rats from both the treated and the control groups were re-anesthetized and a dye was applied below the site of the contusion. After five to seven days the rats were again deeply anesthetized and their brains were excised, processed, and cryosectioned. Sections taken through the red nucleus were inspected and analyzed qualitatively and quantitatively under fluorescent and confocal microscopes. Significantly, more labelled nuclei were seen in the red nuclei of rats treated with anti-MBP T cells (8A) than in the red nuclei of PBS-treated rats (8B). The quantitative differences are shown in the bar graph (8C) and were obtained from animals with scores of 10 and 11 in the T cell treated group and scores of 6 in the control group. The bar graph shows mean \pm SD.

Fig. 9 is a series of photographs showing diffusion-weighted imaging of contused spinal cord treated with anti-MBP T cells. Spinal cords of MBP-T cell-treated and PBS-treated animals (with locomotion scores of 10 and 8, respectively) were excised under deep anesthesia, immediately fixed in 4% paraformaldehyde solution, and placed into 5 mm NMR tubes. Diffusion anisotropy was measured in a Bruker DMX 400 widebore spectrometer using a microscopy probe with a 5-mm Helmholtz coil and actively shielded magnetic field gradients. A multislice pulsed gradient spin echo experiment was performed with 9 axial slices, with the central slice positioned at the center of the spinal injury. Images were acquired with TE of 31 ms, TR of 2000 ms, a diffusion time of 15 ms, a diffusion gradient duration of 3 ms, field of view 0.6 mm, matrix size 128 x 128, slice thickness 0.5 mm, and slice separation of 1.18 mm. Four diffusion gradient values of 0, 28, 49, and 71 g/cm were applied along the read direction (transverse diffusion) or along the slice direction (longitudinal diffusion). Diffusion anisotropy is manifested by increased signal intensity in the images with the highest transverse diffusion gradient relative to the longitudinal diffusion gradient. The excised spinal cords of a PBS-treated rat and in the rat treated with MBP-T cells were subjected to diffusion-weighted MRI analysis. In the PBS-treated injured control, diffusion anisotropy was seen mainly in sections near the proximal and distal stumps of the

cord, with low anisotropy in sections taken through the site of injury. In contrast, in the treated rat, higher levels of diffusion anisotropy can be seen in sections taken through the site of injury.

Fig. 10 is a graph illustrating inhibition of secondary degeneration after optic nerve crush injury in adult rats. See text, Section 8, for experimental details. Rats were injected intradermally through the footpads with a 21-mer peptide based on amino acid residues 35-55 (MOG p35-55) of myelin/oligodendrocyte glycoprotein (chemically synthesized at the Weizmann Institute, Israel) (50μ /animal) or PBS ten days prior to optic nerve crush injury or MOG p35-55 in the absence of crush injury. MOG p35-55 was administered with Incomplete Freund's Adjuvant. Surviving optic nerve fibers were monitored by retrograde labeling of retinal ganglion cells (RGCs). The number of RGCs in rats injected with PBS or MOG p35-55 was expressed as a percentage of the total number of neurons in rats injected with MOG p35-55 in the absence of crush injury.

Fig. 11 is a graph illustrating inhibition in adult rats of secondary degeneration after optic nerve crush injury by MBP. See text, Section 9, for experimental details. MBP (Sigma, Israel) (1 mg in 0.5 ml saline) was administered orally to adult rats by gavage using a blunt needle. MBP was administered 5 times, i.e., every third day beginning two weeks prior to optic nerve crush injury. Surviving optic nerve fibers were monitored by retrograde labeling of retinal ganglion cells (RGCs). The number of RGCs in treated rats was expressed as a percentage of the total number of neurons in untreated rats following the injury.

Figs. 12 (A-F) show expression of B7 costimulatory molecules in intact and injured rat optic nerve. Optic nerves were excised from adult Lewis rats before (12A, 12B) and three days after injury (12C, 12D, 12E) and analyzed immunohistochemically for expression of the B7 costimulatory molecule. The site of injury was delineated by GFAP staining. Using calibrated cross-action forceps, the right optic nerve was subjected to a mild crush injury 1-2 mm from the eye. The uninjured contralateral nerve was left undisturbed. Immunohistochemical analysis of optic nerve antigens was

performed as follows. Briefly, longitudinal cryosections of the excised nerves (20 μm thick) were picked up onto gelatin-coated glass and fixed with ethanol for ten minutes at room temperature. The sections were washed and incubated for one hour at room temperature with mouse monoclonal antibody to rat GFAP (BioMakor, Israel), diluted 1:100, and with antibodies to B7.2 costimulatory molecule and the B7.1 costimulatory molecule (PHARMINGEN, San Diego, CA), diluted 1:25. The sections were washed again and incubated with rhodamine isothiocyanate-conjugated goat anti-mouse IgG (with minimal cross-reaction to rat, human, bovine and horse serum protein) (Jackson ImmunoResearch, West Grove, PA), for one hour at room temperature. All washing solutions contained PBS and 0.05% Tween-20. All diluting solutions contained PBS containing 3% fetal calf serum and 2% bovine serum albumin. The sections were treated with glycerol containing 1,4-diazobicyclo-(2,2,2)-octane and were then viewed with a Zeiss microscope. Note the morphological changes of the B7.2 positive cells after injury, from a rounded (12A, 12B) to a star-like shape (12C, 12D). The B7.2 positive cells were present at a higher density closer to the injury site (12E). Expression of B7.1 was detectable only from day seven and only at the injured site (12F).

Figs. 13 A-C show immunohistochemical analysis of T cells, macrophages or microglia, and B7.2 costimulatory molecules in the injured optic nerves of rats fed MBP. Lewis rats aged 6-8 weeks were fed 1 mg of bovine MBP (Sigma, Israel) (2 mg MBP/ml PBS) or 0.5 ml PBS only every other day by gastric intubation using a stainless steel feeding needle (Thomas Scientific, Swedesboro, NJ) (Chen, Y., Kuchroo, V.K., Inobe, J. Hafler, D.A. & Weiner, H.L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265:1237-1240, 1994). Ten days after starting MBP the right optic nerves were subjected to calibrated crush injury, as described for Figure 12. Three days later the nerves were excised and prepared for immunohistochemical analysis of T cells using mouse monoclonal antibodies to T cell receptor 11, diluted 1:25, macrophages or microglia using anti-ED1 antibodies (Serotek, Oxford, U.K) diluted 1:250, astrocytes using anti-GFAP antibodies and B7.2

costimulatory molecules as described for Fig. 12. There were no significant quantitative differences in T cells or in ED-1 positive cells between injured optic nerves of PBS-fed (13A) and MBP-fed (13B) rats. The number of B7.2 positive cells at the site of injury of MBP-fed rats (13C) should be noted, as compared with injured controls (Fig. 12E).

Fig. 14 is a graph showing the slowing of neuronal degeneration in rats with orally induced tolerance to MBP. Lewis rats were fed 1 mg MBP daily, or every other day, or 4 times a day at two hour intervals for five consecutive days. Control animals were given PBS or the non-self antigen OVA (Sigma, Israel). Ten days after the start of MBP ingestion, the right optic nerves were subjected to a calibrated mild crush injury. Two weeks later the RGCs were retrogradely labelled by application of the fluorescent lipophilic dye, 4-(4-didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Netherlands), distally to the site of injury, as described. Briefly, complete axotomy was performed 1-2 mm from the distal border to the injury site, and solid crystals (0.2-0.4 mm in diameter) of 4-Di-10-Asp were immediately deposited at the site of the lesion. Retrograde labelling of RGCs by the dye gives a reliable indication of the number of still-functioning neurons, as only intact axons can transport the dye to their cell bodies in the retina. Six days after dye application, the retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labelled ganglion cells by fluorescence microscopy. RGCs were counted from three different regions in the retina. The results are expressed as normalized percentage of each retina to untreated injured animal mean of the same experiment. The median of each group is shown as a bar (Control vs. MBP OTx4 ** P<0.01; Control vs. MBP OT ** P, 0.01; Control vs. OVA OT ns P>0.05).

Fig. 15 shows the nucleotide sequence of rat myelin basic protein gene, SEQ ID NO:1, Genbank accession number M25889 (Schaich et al., Biol. Chem. 367:825-834, 1986).

Fig. 16 shows the nucleotide sequence of human myelin basic protein gene, SEQ ID NO:2, Genbank accession number

M13577 (Kamholz et al., Proc. Natl. Acad. Sci. U.S.A. 83(13): 4962-4966, 1986).

Figs 17 (A-F) show the nucleotide sequences of human myelin proteolipid protein gene exons 1-7, SEQ ID NOs:3-8, respectively, Genbank accession number M15026-M15032 respectively (Diehl et al., Proc. Natl. Acad. Sci. U.S.A. 83(24):9807-9811, 1986; published erratum appears in Proc Natl Acad Sci U.S.A. 86(6):617-8, 1991).

Fig. 18 shows the nucleotide sequence of human myelin oligodendrocyte glycoprotein gene, SEQ ID NO:9, Genbank accession number Z48051 (Roth et al., submitted (17-Jan-1995) Roth, CNRS UPR 8291, CIGH, CHU Purpan, Toulouse, France, 31300; Gonzalez et al., Mol. Phylogent. Evol. 6:63-71, 1996).

Fig. 19 shows the nucleotide sequence of rat proteolipid protein and variant, SEQ ID NO:10, Genbank accession number M16471 (Nave et al., Proc. Natl. Acad. Sci. U.S.A. 84:600-604, 1987).

Fig. 20 shows the nucleotide sequence of rat myelin-associated glycoprotein, SEQ ID NO:11, Genbank accession number M14871 (Arquint et al., Proc. Natl. Acad. Sci. USA 84:600-604, 1987).

Fig. 21 shows the amino acid sequence of human myelin basic protein, SEQ ID NO:12, Genbank accession number 307160 (Kamholz et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83(13):4962-4966, 1986).

Fig. 22 shows the amino acid sequence of human proteolipid protein, SEQ ID NO:13, Genbank accession number 387028.

Fig. 23 shows the amino acid sequence of human myelin oligodendrocyte glycoprotein, SEQ ID NO:14, Genbank accession number 793839 (Roth et al., Genomics 28(2):241-250, 1995; Roth Submitted (17-JAN-1995) Roth CNRS UPR 8291, CIGH, CHU Purpan, Toulouse, France, 31300; Gonzalez et al., Mol. Phylogent. Evol. 6:63-71, 1996).

DETAILED DESCRIPTION OF THE INVENTION

Merely for ease of explanation, the detailed description of the present invention is divided into the following subsections: (1) NS-specific activated T cells; (2)

NS-specific antigens, peptides derived therefrom and derivatives thereof; (3) nucleotide sequences encoding NS-specific antigens and peptides derived therefrom; (4) therapeutic uses of non-recombinant, NS-specific activated T cells, NS-specific antigens, peptides derived therefrom and derivatives thereof, and nucleotide sequences encoding NS-specific antigens and peptides derived therefrom; and (5) formulations and modes of administration of nonrecombinant, NS-specific activated T cells, NS-specific antigens, peptides derived therefrom and derivatives thereof, and nucleotide sequences encoding NS-specific antigens and peptides derived therefrom.

5.1 NS-SPECIFIC ACTIVATED T CELLS

NS-specific activated T cells (ATCs) can be used for ameliorating or inhibiting the effects of injury or disease of the CNS or PNS that result in NS degeneration or for promoting regeneration in the NS, in particular the CNS.

The NS-specific activated T cells are preferably autologous, most preferably of the CD4 and/or CD8 phenotypes, but they may also be allogeneic T cells from related donors, e.g., siblings, parents, children, or HLA-matched or partially matched, semi-allogeneic or fully allogeneic donors.

In addition to the use of autologous T cells isolated from the subject, the present invention also comprehends the use of semi-allogeneic T cells for neuroprotection. These T cells may be prepared as short- or long-term lines and stored by conventional cryopreservation methods for thawing and administration, either immediately or after culturing for 1-3 days, to a subject suffering from injury to the central nervous system and in need of T cell neuroprotection.

The use of semi-allogeneic T cells is based on the fact that T cells can recognize a specific antigen epitope presented by foreign antigen presenting cells (APC), provided that the APC express the MHC molecule, class I or class II, to which the specific responding T cell population is restricted, along with the antigen epitope recognized by the T cells. Thus, a semi-allogeneic population of T cells that can recognize at least one allelic product of the subject's MHC

molecules, preferably an HLA-DR or an HLA-DQ or other HLA molecule, and that is specific for a NS-associated antigen epitope, will be able to recognize the NS antigen in the subject's area of NS damage and produce the needed neuroprotective effect. There is little or no polymorphism in the adhesion molecules, leukocyte migration molecules, and accessory molecules needed for the T cells to migrate to the area of damage, accumulate there, and undergo activation. Thus, the semi-allogeneic T cells will be able to migrate and accumulate at the CNS site in need of neuroprotection and will be activated to produce the desired effect.

It is known that semi-allogeneic T cells will be rejected by the subject's immune system, but that rejection requires about two weeks to develop. Hence, the semi-allogeneic T cells will have the two week window of opportunity needed to exert neuroprotection. After two weeks, the semi-allogeneic T cells will be rejected from the body of the subject, but that rejection is advantageous to the subject because it will rid the subject of the foreign T cells and prevent any untoward consequences of the activated T cells. The semi-allogeneic T cells thus provide an important safety factor and are a preferred embodiment.

It is known that a relatively small number of HLA class II molecules are shared by most individuals in a population. For example, about 50% of the Jewish population express the HLA-DR5 gene. Thus, a bank of specific T cells reactive to NS antigen epitopes that are restricted to HLA-DR5 would be useful in 50% of that population. The entire population can be covered essentially by a small number of additional T cell lines restricted to a few other prevalent HLA molecules, such as DR1, DR4, DR2, etc. Thus, a functional bank of uniform T cell lines can be prepared and stored for immediate use in almost any individual in a given population. Such a bank of T cells would overcome any technical problems in obtaining a sufficient number of specific T cells from the subject in need of neuroprotection during the open window of treatment opportunity. The semi-allogeneic T cells will be safely rejected after accomplishing their role of neuroprotection. This aspect of the invention does not

contradict, and is in addition to the use of autologous T cells as described herein.

The NS-specific activated T cells are preferably non-attenuated, although attenuated NS-specific activated T cells may be used. T cells may be attenuated using methods well known in the art, including but not limited to, by gamma-irradiation, e.g., 1.5-10.0 Rads (Ben-Nun, A., Wekerle, H. and Cohen, I.R., Nature 292:60-61 (1981); Ben-Nun, A. and Cohen, I.R., J. Immunol. 129:303-308 (1982)); and/or by pressure treatment, for example as described in U.S. Patent No. 4,996,194 (Cohen et al.); and/or by chemical cross-linking with an agent such as formaldehyde, glutaraldehyde and the like, for example as described in U.S. Patent No. 4,996,194 (Cohen et al.); and/or by cross-linking and photoactivation with light with a photoactivatable psoralen compound, for example as described in U.S. Patent No. 5,114,721 (Cohen et al.); and/or by a cytoskeletal disrupting agent such as cytochalsin and colchicine, for example as described in U.S. Patent No. 4,996,194 (Cohen et al.). In a preferred embodiment the NS-specific activated T cells are isolated as described below. T cells can be isolated and purified according to methods known in the art (Mor and Cohen, 1995, J. Immunol. 155:3693-3699). For an illustrative example, see Section 6.1.

Circulating T cells of a subject which recognize myelin basic protein or another NS antigen, such as the amyloid precursor protein, are isolated and expanded using known procedures. In order to obtain NS-specific activated T cells, T cells are isolated and the NS-specific ATCs are then expanded by a known procedure (Burns et al., Cell Immunol. 81:435, 1983; Pette et al., Proc. Natl. Acad. Sci. USA 87:7968, 1990; Mortin et al., J. Immunol. 145:540, 1990; Schluesener et al., J. Immunol. 135:3128, 1985; Suruhan-Dires Keneli et al., Euro. J. Immunol. 23:530, 1993, which are incorporated herein by reference in their entirety).

The isolated T cells may be activated by exposure of the cells to one or more of a variety of natural or synthetic NS-specific antigens or epitopes, including but not limited to, myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated

glycoprotein (MAG), S-100, β -amyloid, Thy-1, P0, P2 and neurotransmitter receptors. In a preferred embodiment, the isolated T cells are activated by one or more cryptic epitopes, including but limited to the following MBP peptides: p11-30, p51-70, p91-110, p131-150, and p-151-170.

During *ex vivo* activation of the T cells, the T cells may be activated by culturing them in medium to which at least one suitable growth promoting factor has been added. Growth promoting factors suitable for this purpose include, without limitation, cytokines, for instance tumor necrosis factor α (TNF- α), interleukin 2 (IL-2), and interleukin 4 (IL-4).

In one embodiment, the activated T cells endogenously produce a substance that ameliorates the effects of injury or disease in the NS.

In another embodiment, the activated T cells endogenously produce a substance that stimulates other cells, including, but not limited to, transforming growth factor- β (TGF- β), nerve growth factor (NGF), neurotrophic factor 3 (NT-3), neurotrophic factor 4/5 (NT-4/5), brain derived neurotrophic factor (BDNF); interferon- γ (IFN- γ), and interleukin-6 (IL-6), wherein the other cells, directly or indirectly, ameliorate the effects of injury or disease.

Following their proliferation *in vitro*, the T cells are administered to a mammalian subject. In a preferred embodiment, the T cells are administered to a human subject. T cell expansion is preferably performed using peptides corresponding to sequences in a non-pathogenic, NS-specific, self protein.

A subject can initially be immunized with an NS-specific antigen using a non-pathogenic peptide of the self protein. A T cell preparation can be prepared from the blood of such immunized subjects, preferably from T cells selected for their specificity towards the NS-specific antigen. The selected T cells can then be stimulated to produce a T cell line specific to the self-antigen (Ben-Nun et al., J. Immunol. 129:303, 1982).

The NS-specific antigen may be a purified antigen or a crude NS preparation, as will be described below. NS-

specific antigen activated T cells, obtained as described above, can be used immediately or may be preserved for later use, e.g., by cryopreservation as described below. NS-specific activated T cells may also be obtained using previously cryopreserved T cells, i.e., after thawing the cells, the T cells may be incubated with NS-specific antigen, optimally together with thymocytes, to obtain a preparation of NS-specific ATCs.

As will be evident to those skilled in the art, the T cells can be preserved, e.g., by cryopreservation, either before or after culture.

Cryopreservation agents which can be used include but are not limited to dimethyl sulfoxide (DMSO) (Lovelock and Bishop, Nature 183:1394-1395, 1959; Ashwood-Smith, Nature 190:1204-1205, 1961), glycerol, polyvinylpyrrolidone (Rinfret, Ann. N.Y. Acad. Sci. 85:576, 1960), polyethylene glycol (Sloviter and Ravdin, Nature 196:548, 1962), albumin, dextran, sucrose, ethylene glycol, i-erythritol, D-ribitol, D-mannitol (Rowe et al., Fed. Proc. 21:157, 1962), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender et al., J. Appl. Physiol. 15:520, 1960), amino acids (Phan The Tran and Bender, Exp. Cell Res. 20:651, 1960), methanol, acetamide, glycerol monoacetate (Lovelock, Biochem. J. 56:265, 1954), inorganic salts (Phan The Tran and Bender, Proc. Soc. Exp. Biol. Med. 104:388, 1960; Phan The Tran and Bender, 1961, in Radiobiology, Proceedings of the Third Australian Conference on Radiobiology, Ilbery, P.L.T., ed., Butterworth, London, p. 59), and DMSO combined with hydroxyethyl starch and human serum albumin (Zaroulis and Leiderman, Cryobiology 17:311-317, 1980).

A controlled cooling rate is critical. Different cryoprotective agents (Rapatz et al., Cryobiology 5(1):18-25, 1968) and different cell types have different optimal cooling rates. See, e.g., Rowe and Rinfret, Blood 20:636 (1962); Rowe, Cryobiology 3(1):12-18 (1966); Lewis et al., Transfusion 7(1):17-32 (1967); and Mazur, Science 168:939-949 (1970) for effects of cooling velocity on survival of cells and on their transplantation potential. The heat of fusion phase where water turns to ice should be minimal. The cooling procedure

can be carried out by use of, e.g., a programmable freezing device or a methanol bath procedure.

Programmable freezing apparatuses allow determination of optimal cooling rates and facilitate standard reproducible cooling. Programmable controlled-rate freezers such as Cryomed or Planar permit tuning of the freezing regimen to the desired cooling rate curve.

After thorough freezing, cells can be rapidly transferred to a long-term cryogenic storage vessel. In one embodiment, samples can be cryogenically stored in mechanical freezers, such as freezers that maintain a temperature of about -80°C or about -20°C. In a preferred embodiment, samples can be cryogenically stored in liquid nitrogen (-196°C) or its vapor. Such storage is greatly facilitated by the availability of highly efficient liquid nitrogen refrigerators, which resemble large Thermos containers with an extremely low vacuum and internal super insulation, such that heat leakage and nitrogen losses are kept to an absolute minimum.

Considerations and procedures for the manipulation, cryopreservation, and long term storage of T cells can be found, for example, in the following references, incorporated by reference herein: Gorin, Clinics in Haematology 15(1):19-48 (1986); Bone-Marrow Conservation, Culture and Transplantation, Proceedings of a Panel, Moscow, July 22-26, 1968, International Atomic Energy Agency, Vienna, pp. 107-186.

Other methods of cryopreservation of viable cells, or modifications thereof, are available and envisioned for use, e.g., cold metal-mirror techniques. See Livesey and Linner, Nature 327:255 (1987); Linner et al., J. Histochem. Cytochem. 34(9):1123-1135 (1986); see also U.S. Patent No. 4,199,022 by Senken et al., U.S. Patent No. 3,753,357 by Schwartz, U.S. Patent No. 4,559,298 by Fahy.

Frozen cells are preferably thawed quickly (e.g., in a water bath maintained at 37-47°C) and chilled immediately upon thawing. It may be desirable to treat the cells in order to prevent cellular clumping upon thawing. To prevent clumping, various procedures can be used, including but not limited to the addition before or after freezing of DNase (Spitzer et al., Cancer 45:3075-3085, 1980), low molecular

weight dextran and citrate, citrate, hydroxyethyl starch (Stiff et al., Cryobiology 20:17-24, 1983), or acid citrate dextrose (Zaroulis and Leiderman, Cryobiology 17:311-317, 1980), etc.

The cryoprotective agent, if toxic in humans, should be removed prior to therapeutic use of the thawed T cells. One way in which to remove the cryoprotective agent is by dilution to an insignificant concentration.

Once frozen T cells have been thawed and recovered, they are used to promote neuronal regeneration as described herein with respect to non-frozen T cells. Once thawed, the T cells may be used immediately, assuming that they were activated prior to freezing. Preferably, however, the thawed cells are cultured before injection to the patient in order to eliminate non-viable cells. Furthermore, in the course of this culturing over a period of about one to three days, an appropriate activating agent can be added so as to activate the cells, if the frozen cells were resting T cells, or to help the cells achieve a higher rate of activation if they were activated prior to freezing. Usually, time is available to allow such a culturing step prior to administration as the T cells may be administered as long as a week after injury, and possibly longer, and still maintain their neuroregenerative and neuroprotective effect.

5.2 NS-SPECIFIC ANTIGENS AND PEPTIDES DERIVED THEREFROM

Pharmaceutical compositions comprising an NS-specific antigen or peptide derived therefrom or derivative thereof can be used for preventing or inhibiting the effects of injury or disease that result in NS degeneration or for promoting nerve regeneration in the NS, particularly in the CNS. Additionally, NS-specific antigens or peptides derived therefrom or derivatives thereof may be used for *in vivo* or *in vitro* activation of T cells. In one embodiment, the NS-specific antigen is an isolated or purified antigen. In another embodiment, methods of promoting nerve regeneration or of preventing or inhibiting the effects of CNS or PNS injury or disease comprise administering NS-specific antigen or a peptide derived therefrom or derivative thereof to a mammal wherein the

NS-specific antigen or peptide derived therefrom or derivative thereof activates T cells *in vivo* to produce a population of T cells that accumulate at a site of injury or disease of the CNS or PNS.

The NS-specific antigen may be an antigen obtained from NS tissue, preferably from tissue at a site of CNS injury or disease. The NS-specific antigen may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of antigens. The functional properties may be evaluated using any suitable assay. In the practice of the invention, natural or synthetic NS-specific antigens or epitopes include, but are not limited to, MBP, MOG, PLP, MAG, S-100, β -amyloid, Thy-1, P0, P2 and a neurotransmitter receptor.

Specific illustrative examples of useful NS-specific antigens include but are not limited to, human MBP, depicted in Fig. 21, (SEQ ID NO:12); human proteolipid protein, depicted in Fig. 22 (SEQ ID NO:13); and human oligodendrocyte glycoprotein, depicted in Fig. 23 (SEQ ID NO:14).

In a preferred embodiment, peptides derived from NS-specific, self-antigens or derivatives of NS-specific antigens activate T cells, but do not induce an autoimmune disease. An example of such peptide is a peptide comprising amino acids 51-70 of myelin basic protein (residues 51-70 of SEQ ID NO:12).

In addition, an NS-specific antigen may be a crude NS-tissue preparation, e.g., derived from NS tissue obtained from mammalian NS. Such a preparation may include cells, both living or dead cells, membrane fractions of such cells or tissue, etc.

An NS-specific antigen may be obtained by an NS biopsy or necropsy from a mammal including, but not limited to, from a site of CNS injury; from cadavers; from cell lines grown in culture. Additionally, an NS-specific antigen may be a protein obtained by genetic engineering, chemically synthesized, etc.

In addition to NS-specific antigens, the invention also relates to peptides derived from NS-specific antigens or

derivatives including chemical derivatives and analogs of NS-specific antigens which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length NS-specific antigen. Such functional activities include but are not limited to antigenicity (ability to bind (or compete with an NS-antigen for binding) to an anti-NS-specific antibody), immunogenicity (ability to generate antibody which binds to an NS-specific protein), and ability to interact with T cells, resulting in activation comparable to that obtained using the corresponding full-length antigen. The crucial test is that the antigen which is used for activating the T cells causes the T cells to be capable of recognizing an antigen in the NS of the mammal (patient) being treated.

A peptide derived from a CNS-specific or PNS-specific antigen preferably has a sequence comprised within the antigen sequence and is either: (1) an immunogenic peptide, i.e., a peptide that can elicit a human T cell response detected by a T cell proliferation or by cytokine (e.g. interferon (IFN)- γ , interleukin (IL)-2, IL-4 or IL-10) production or (2) a "cryptic epitope" (also designated herein as "immunosilent" or "nonimmunodominant" epitope), i.e., a peptide that by itself can induce a T cell immune response that is not induced by the whole antigen protein (see Moalem et al., Nature Med. 5(1), 1999). Cryptic epitopes for use in the present invention include, but are not limited to, peptides of the myelin basic protein sequence: peptide p11-30, p51-70, p91-110, p131-150, and p151-170. Other peptides can be identified by their capacity to elicit a human T cell response detected by T cell proliferation or by cytokine (e.g. IFN- γ , IL-2, IL-4, or IL-10) production. Such cryptic epitopes are particularly preferred as T cells activated thereby will accumulate at the injury site, in accordance with the present invention, but are particularly weak in autoimmunity. Thus, they would be expected to have fewer side effects.

In one specific embodiment of the invention, peptides consisting of or comprising a fragment of an NS-specific antigen consisting of at least 10 (contiguous) amino acids of the NS-specific antigen are provided. In other embodiments, the

fragment consists of at least 20 contiguous amino acids or 50 contiguous amino acids of the NS-specific antigen. Derivatives of an NS-specific antigen also include but are not limited to those molecules comprising regions that are substantially homologous to the full-length antigen or fragments thereof (e.g., in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding nucleotide sequence of the full-length NS-specific antigen, under high stringency, moderate stringency, or low stringency conditions.

Computer programs for determining homology may include but are not limited to TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85(8):2444-8, 1988; Altschul et al., J. Mol. Biol. 215(3):40310, 1990; Thompson, et al., Nucleic Acids Res. 22(22):4673-80, 1994; Higgins, et al., Methods Enzymol. 266:383-402, 1996; Altschul, et al., 1990, J. Mol. Biol. 215(3):403-410, 1990).

The NS-specific antigen derivatives of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*.

Additionally, the coding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to,

chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., J. Biol. Chem. 253:6551, 1978), etc.

Manipulations may also be made at the protein level. Included within the scope of the invention are derivatives which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, derivatives of an NS-specific antigen can be chemically synthesized. For example, a peptide corresponding to a portion of an antigen which comprises the desired domain or which mediates the desired activity can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acids analogs can be introduced as a substitution or addition into the amino acid sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid; 4-aminobutyric acid, Abu; 2-amino butyric acid, γ -Abu; ϵ -Ahx, 6-amino hexanoic acid; Aib, 2-amino isobutyric acid; 3-amino propionic acid; ornithine; norleucine; novaline; hydroxyproline; sarcosine; citrulline; cysteic acid; t-butylglycine; t-butylalanine; phenylglycine; cyclohexylalanine; β -alanine; fluoro-amino acids; designer amino acids such as β -methyl amino acids, α -methyl amino acids, $N\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

The functional activity of NS-specific antigens and peptides derived therefrom and derivatives thereof can be assayed by various methods known in the art, including, but not limited to, T cell proliferation assays (Mor and Cohen, J. Immunol. 155:3693-3699, 1995).

An NS-specific antigen or peptide derived therefrom or derivative thereof may be kept in solution or may be provided in a dry form, e.g. as a powder or lyophilizate, to be mixed with appropriate solution prior to use.

5.3 NUCLEOTIDE SEQUENCES ENCODING NS-ANTIGENS AND PEPTIDES DERIVED THEREFROM

Compositions comprising a nucleotide sequence encoding an NS-specific antigen or peptide derived therefrom can be used for preventing or inhibiting the effects of injury or disease that result in CNS or PNS degeneration or for promoting nerve regeneration in the CNS or PNS. Specific illustrative examples of useful nucleotide sequences encoding NS-specific antigens or peptides derived from an NS-specific antigen, include but are not limited to nucleotide sequences encoding rat myelin basic protein (MBP) peptides, depicted in Fig. 15 (SEQ ID NO:1); human MBP, depicted in Fig. 16 (SEQ ID NO:2); human myelin PLP, depicted in Figs. 17(A-F) (SEQ ID NOs:3-8); human MOG, depicted in Fig. 18 (SEQ ID NO:9); rat PLP and variant, depicted in Fig. 19 (SEQ ID NO:10); and rat MAG, depicted in Fig. 20 (SEQ ID NO:11).

5.4 THERAPEUTIC USES

The compositions described in Sections 5.1 through 5.3 may be used to promote nerve regeneration or to prevent or inhibit secondary degeneration which may otherwise follow primary NS injury, e.g., blunt trauma, penetrating trauma, hemorrhagic stroke, ischemic stroke or damages caused by surgery such as tumor excision. In addition, such compositions may be used to ameliorate the effects of disease that result in a degenerative process, e.g., degeneration occurring in either grey or white matter (or both) as a result of various diseases or disorders, including, without limitation: diabetic neuropathy, senile dementias, Alzheimer's disease, Parkinson's Disease, facial nerve (Bell's) palsy, glaucoma, Huntington's chorea, amyotrophic lateral sclerosis (ALS), non-arteritic optic neuropathy, intervertebral disc herniation, vitamin deficiency, prion diseases such as Creutzfeldt-Jakob disease, carpal tunnel syndrome, peripheral neuropathies associated with

various diseases, including but not limited to, uremia, porphyria, hypoglycemia, Sjorgren Larsson syndrome, acute sensory neuropathy, chronic ataxic neuropathy, biliary cirrhosis, primary amyloidosis, obstructive lung diseases, acromegaly, malabsorption syndromes, polycythemia vera, IgA and IgG gammopathies, complications of various drugs (e.g., metronidazole) and toxins (e.g., alcohol or organophosphates), Charcot-Marie-Tooth disease, ataxia telangiectasia, Friedreich's ataxia, amyloid polyneuropathies, adrenomyeloneuropathy, Giant axonal neuropathy, Refsum's disease, Fabry's disease, lipoproteinemia, etc.

In a preferred embodiment, the NS-specific activated T cells, the NS-specific antigens, peptides derived therefrom, derivatives thereof or the nucleotides encoding said antigens, or peptides or any combination thereof of the present invention are used to treat diseases or disorders where promotion of nerve regeneration or prevention or inhibition of secondary neural degeneration is indicated, which are not autoimmune diseases or neoplasias. In a preferred embodiment, the compositions of the present invention are administered to a human subject.

While activated NS-specific T cells may have been used in the prior art in the course of treatment to develop tolerance to autoimmune antigens in the treatment of autoimmune diseases, or in the course of immunotherapy in the treatment of NS neoplasms, the present invention can also be used to ameliorate the degenerative process caused by autoimmune diseases or neoplasms as long as it is used in a manner not suggested by such prior art methods. Thus, for example, T cells activated by an autoimmune antigen have been suggested for use to create tolerance to the autoimmune antigen and, thus, ameliorate the autoimmune disease. Such treatment, however, would not have suggested the use of T cells directed to other NS antigens or NS antigens which will not induce tolerance to the autoimmune antigen or T cells which are administered in such a way as to avoid creation of tolerance. Similarly, for neoplasms, the effects of the present invention can be obtained without using immunotherapy processes suggested in the prior art by, for example, using an NS antigen which

does not appear in the neoplasm. T cells activated with such an antigen will still accumulate at the site of neural degeneration and facilitate inhibition of this degeneration, even though it will not serve as immunotherapy for the tumor *per se*.

5.5 FORMULATIONS AND ADMINISTRATION

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. The carriers in the pharmaceutical composition may comprise a binder, such as microcrystalline cellulose, polyvinylpyrrolidone (polyvidone or povidone), gum tragacanth, gelatin, starch, lactose or lactose monohydrate; a disintegrating agent, such as alginic acid, maize starch and the like; a lubricant or surfactant, such as magnesium stearate, or sodium lauryl sulphate; a glidant, such as colloidal silicon dioxide; a sweetening agent, such as sucrose or saccharin; and/or a flavoring agent, such as peppermint, methyl salicylate, or orange flavoring.

Methods of administration include, but are not limited to, parenteral, e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, mucosal (e.g., oral, intranasal, buccal, vaginal, rectal, intraocular), intrathecal, topical and intradermal routes. Administration can be systemic or local.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil,

oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compositions may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen free water, before use.

The compositions may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

For administration by inhalation, the compositions for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized

aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin, for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

In a preferred embodiment, compositions comprising NS-specific activated T cells, an NS-specific antigen or peptide derived therefrom, or derivative thereof, or a nucleotide sequence encoding such antigen or peptide, are formulated in accordance with routine procedures as pharmaceutical compositions adapted for intravenous or intraperitoneal administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water or saline for injection can be provided so that the ingredients may be mixed prior to administration.

Pharmaceutical compositions comprising NS-specific antigen or peptide derived therefrom or derivative thereof may optionally be administered with an adjuvant, such as Incomplete Freund's Adjuvant.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention.

In a preferred embodiment, the pharmaceutical compositions of the invention are administered to a mammal, preferably a human, shortly after injury or detection of a degenerative lesion in the NS. The therapeutic methods of the invention may comprise administration of an NS-specific activated T cell or an NS-specific antigen or peptide derived therefrom or derivative thereof, or a nucleotide sequence encoding such antigen or peptide, or any combination thereof.

When using combination therapy, the NS-specific antigen may be administered before, concurrently or after administration of NS-specific activated T cells, a peptide derived from an NS-specific antigen or derivative thereof or a nucleotide sequence encoding such antigen or peptide.

In one embodiment, the compositions of the invention are administered in combination with one or more of the following (a) mononuclear phagocytes, preferably cultured monocytes (as described in PCT publication No. WO 97/09985, which is incorporated herein by reference in its entirety), that have been stimulated to enhance their capacity to promote neuronal regeneration; (b) a neurotrophic factor such as acidic fibroblast growth factor; and (c) an anti-inflammatory therapeutic substance (i.e., an anti-inflammatory steroid, such as dexamethasone or methylprednisolone, or a non-steroidal anti-inflammatory peptide, such as Thr-Lys-Pro (TKP)).

In another embodiment, mononuclear phagocyte cells according to PCT Publication No. WO 97/09985 and U.S. patent application Serial No. 09/041,280, filed March 11, 1998, are injected into the site of injury or lesion within the CNS, either concurrently, prior to, or following parenteral administration of NS-specific activated T cells, an NS-specific antigen or peptide derived therefrom or derivative thereof, or a nucleotide sequence encoding such antigen or peptide

In another embodiment, administration of NS-specific activated T cells, NS-specific antigen or peptide sequence encoding such antigen or peptide, may be administered as a single dose or may be repeated, preferably at 2 week intervals and then at successively longer intervals once a month, once a quarter, once every six months, etc. The course of treatment may last several months, several years or occasionally also through the life-time of the individual, depending on the condition or disease which is being treated. In the case of a CNS injury, the treatment may range between several days to months or even years, until the condition has stabilized and there is no or only a limited risk of development of secondary degeneration. In chronic human disease or Parkinson's disease, the therapeutic treatment in accordance with the invention may be for life.

As will be evident to those skilled in the art, the therapeutic effect depends at times on the condition or disease to be treated, on the individual's age and health condition, on other physical parameters (e.g. gender, weight, etc.) of the individual, as well as on various other factors, e.g., whether the individual is taking other drugs, etc.

The optimal dose of the therapeutic compositions comprising NS-specific activated T cells of the invention is proportional to the number of nerve fibers affected by NS injury or disease at the site being treated. In a preferred embodiment, the dose ranges from about 5×10^6 to about 10^7 for treating a lesion affecting about 10^5 nerve fibers, such as a complete transection of a rat optic nerve, and ranges from about 10^7 to about 10^8 for treating a lesion affecting about 10^6 - 10^7 nerve fibers, such as a complete transection of a human optic nerve. As will be evident to those skilled in the art, the dose of T cells can be scaled up or down in proportion to the number of nerve fibers thought to be affected at the lesion or site of injury being treated.

5.6 ESTABLISHMENT OF AUTOLOGOUS CELL BANKS FOR T LYMPHOCYTES

To minimize secondary damage after nerve injury, patients can be treated by administering autologous or semi-allogeneic T lymphocytes sensitized to at least one appropriate NS antigen. As the window of opportunity has not yet been precisely defined, therapy should be administered as soon as possible after the primary injury to maximize the chances of success, preferably within about one week.

To bridge the gap between the time required for activation and the time needed for treatment, a bank can be established with personal vaults of autologous T lymphocytes prepared for future use for neuroprotective therapy against secondary degeneration in case of NS injury. T lymphocytes are isolated from the blood and then sensitized to a NS antigen. The cells are then frozen and suitably stored under the person's name, identity number, and blood group, in a cell bank until needed.

Additionally, autologous stem cells of the CNS can be processed and stored for potential use by an individual patient in the event of traumatic disorders of the NS such as ischemia or mechanical injury, as well as for treated neurodegenerative conditions such as Alzheimer's disease or Parkinson's disease. Alternatively, semi-allogeneic or allogeneic T cells can be stored frozen in banks for use by any individual who shares one MHC type II molecule with the source of the T cells.

The following examples illustrate certain features of the present invention but are not intended to limit the scope of the present invention.

**EXAMPLE: ACCUMULATION OF ACTIVATED T CELLS IN INJURED
OPTIC NERVE**

6.1 MATERIALS AND METHODS

6.1.1 ANIMALS

Female Lewis rats were supplied by the Animal Breeding Center of the Weizmann Institute of Science (Rehovot, IL), matched for age (8-12 weeks) and housed four to a cage in a light and temperature-controlled room.

6.1.2 MEDIA

The T cell proliferation medium contained the following: Dulbecco's modified Eagle's medium (DMEM, Biological 15 Industries, Israel) supplemented with 2mM L-glutamine (L-Glu, Sigma, USA), 5×10^{-5} M 2-mercaptoethanol (2-ME, Sigma), penicillin (100 IU/ml; Biological Industries), streptomycin (100 μ /ml; Biological Industries), sodium pyruvate (1 mM; Biological Industries), non-essential amino acids (1 ml/100 ml; Biological Industries) and autologous rat serum 1% (vol/vol) (Mor et al., *Clin. Invest.* 85:1594, 1990). Propagation medium contained: DMEM, 2-ME, L-Glu, sodium pyruvate, non-essential amino acids and antibiotics in the same concentration as above with the addition of 10% fetal calf serum (FCS), and 10% T cell growth factor (TCGF) obtained from the supernatant of concanavalin A-stimulated spleen cells (Mor et al., *supra*, 1990).

6.1.3 ANTIGENS

Myelin basic protein (MBP) from the spinal cords of guinea pigs was prepared as described (Hirshfeld, et al., FEBS Lett. 7:317, 1970). Ovalbumin was purchased from Sigma (St. Louis, Missouri). The p51-70 of the rat 18.5kDa isoform of MBP (sequence: APKRGSGKDSHTRTTHYG) (SEQ ID NO:15) and the p277 peptide of the human hsp60 (sequence: VLGGGCALLRCPALDSLTPANED) (SEQ ID NO:16) (Elias et al., Proc. Natl. Acad. Sci. USA 88:3088-3091, 1991) were synthesized using the 9-fluorenylmethoxycarbonyl technique with an automatic multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany). The purity of the peptides was analyzed by HPLC and amino acid composition.

6.1.4 T CELL LINES

T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with an antigen (described above in Section 6.1.3). The antigen was dissolved in PBS (1mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, Michigan) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco 15 Laboratories, Detroit, Michigan). The emulsion (0.1 ml) was injected into hind foot pads of the rats. Ten days after the antigen was injected, the rats were killed and draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 µg/ml) in proliferation medium (described above in Section 6.1.2). After incubation for 72 h at 37°C, 90% relative humidity and 7% CO₂, the cells were transferred to propagation medium (described above in Section 6.1.2). Cells were grown in propagation medium for 4-10 days before being re-exposed to antigen (10 µg/ml) in the presence of irradiated (2000 red) thymus cells (10⁷ cells/ml) in proliferation medium. The T cell lines were expanded by repeated re-exposure and propagation.

6.1.5 CRUSH INJURY OF RAT OPTIC NERVE

Crush injury of the optic nerve was performed as

previously described (Duvdevani et al., Neurol. Neurosci. 2:31-38, 1990). Briefly, rats were deeply anesthetized by i.p. injection of Rompum (xylazine, 10 mg/kg; Vitamed, Israel) and Vetaler (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, Iowa). Using a binocular operating microscope, a lateral canthotomy was performed in the right eye and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, a moderate crush injury was inflicted on the optic nerve, 2mm from the eye (Duvdevani et al., Instructure Neurology and Neuroscience 2:31, 1990). The contralateral nerve was left undisturbed and was used as a control.

6.1.6 IMMUNOCYTOCHEMISTRY OF T CELLS

Longitudinal cryostat nerve sections (20 μm thick) were picked up onto gelatin glass slides and frozen until preparation for fluorescent staining. Sections were thawed and fixed in ethanol for 10 minutes at room temperature, washed twice with double-distilled water (ddH_2O), and incubated for 3 minutes in PBS containing 0.05% polyoxyethylene-sorbitan monolaurate (Tween-20; Sigma, USA). Sections were then incubated for 1 hr at room temperature with a mouse monoclonal antibody directed against rat T cell receptor (TCR) (1:100, Hunig et al., J. Exp. Med., 169:73, 1989), in PBS containing 3% FCS and 2% BSA. After three washes with PBS containing 0.05% Tween-20, the sections were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (with minimal cross-section to rat, human, bovine and horse serum proteins) (Jackson ImmunoResearch, West Grove, Pennsylvania) for one hour at room temperature. The sections were then washed with PBS containing Tween-20 and treated with glycerol containing 1,4-diazobicyclo-(2,2,2) octane (Sigma), to inhibit quenching of fluorescence. The sections were viewed with a Zeis microscope and cells were counted. Staining in the absence of first antibody was negative.

6.2. RESULTS

Fig. 1 shows accumulation of T cells measured immunohistochemically. The number of T cells was considerably higher in injured nerves rats injected with anti-MBP, anti-OVA or anti-p277 cells; statistical analysis (one-way ANOVA) showed significant differences between T cell numbers in injured optic nerves of rats injected with ant-MBP, anti-OVA, or anti-p277 T cells and in injured optic nerves of rats injected with PBS ($P<0.001$); and between injured optic nerves and uninjured optic nerves of rats injected with anti-MBP, anti-OVA, or anti-p277 T cells ($P<0.001$).

EXAMPLE: NEURPROTECTION BY AUTOIMMUNE ANTI-MBP T CELLS

7.1 MATERIAL AND METHODS

Animals, media, antigens, crush injury of rat optic nerve, sectioning of nerves, T cell lines, and immunolabeling of nerve sections are described in Section 6, *supra*.

7.1.1. RETROGRADE LABELING AND MEASUREMENT OF PRIMARY DAMAGE AND SECONDARY DEGENERATION

Primary damage of the optic nerve axons and their attached retinal ganglion cells (RGCs) were measured after the immediate post-injury application of the fluorescent lipophilic dye 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-Asp) (Molecular Probes Europe BV, Netherlands) distal to the site of injury. Only axons that are intact are capable of transporting the dye back to their cell bodies; therefore, the number of labeled cell bodies is a measure of the number of axons that survived the primary damage. Secondary degeneration was also measured by application of the dye distal to the injury site, but two weeks after the primary lesion was inflicted. Application of the neurotracer dye distal to the site of the primary crush after two weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach makes it possible to differentiate between neurons that are still functionally intact and neurons in which the axons are injured but the cell

bodies are still viable, as only those neurons whose fibers are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labeled ganglion cells reliably reflects the number of still-functioning neurons. Labeling and measurement were done by exposing the right optic nerve for a second time, again without damaging the retinal blood supply. Complete axotomy was done 1-2 mm from the distal border of the injury site and solid crystals (0.2-0.4 mm in diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Uninjured optic nerves were similarly labeled at approximately the same distance from the globe. Five days after dye application, the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution and examined for labeled ganglion cells by fluorescence microscopy. The percentage of RGCs surviving secondary degeneration was calculated using the following formula: (Number of spared neurons after secondary degeneration)/(Number of spared neurons after primary damage) x 100.

7.1.2 ELECTROPHYSIOLOGICAL RECORDINGS

Nerves were excised and their compound action potentials (CAPs) were recorded *in vitro* using a suction electrode experimental set-up (Yoles et al., *J. Neurotrauma* 13:49-57, 1996). At different times after injury and injection of T cells or PBS, rats were killed by intraperitoneal injection of pentobarbitone (170 mg/kg) (CTS Chemical Industries, Israel). Both optic nerves were removed while still attached to the optic chiasma, and were immediately transferred to a vial containing a fresh salt solution consisting of 126 mM NaCl, 3 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 2 mM MgSO₄, 2 mM CaCl₂, and 10 mM D-glucose, aerated with 95% O₂ and 5% CO₂ at room temperature. After 1 hour, electrophysiological recordings were made. In the injured nerve, recordings were made in a segment distal to the injury site. This segment contains axons of viable retinal ganglion cells that have escaped both primary and secondary damage, as well as the distal stumps of non-viable retinal ganglion cells

that have not yet undergone Wallerian degeneration. The nerve ends were connected to two suction Ag-AgCl electrodes immersed in the bathing solution at 37°C. A stimulating pulse was applied through the electrode, and the CAP was recorded by the distal electrode. A stimulator (SD9; Grass Medical Instruments, Quincy, Massachusetts) was used for supramaximal electrical stimulation at a rate of 1 pps to ensure stimulation of all propagating axons in the nerve. The measured signal was transmitted to a microelectrode AC amplifier (model 1800; A-M Systems, Everett, Washington). The data were processed using the LabView 2.1.1 data acquisition and management system (National Instruments, Austin, Texas). For each nerve, the difference between the peak amplitude and the mean plateau of eight CAPs was computed and was considered as proportional to the number of propagating axons in the optic nerve. The experiments were done by experimentors "blinded", to sample identity. In each experiment the data were normalized relative to the mean CAP of the uninjured nerves from PBS-injected rats,

7.1.3 CLINICAL EVALUATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Clinical disease was scored every 1 to 2 days according to the following neurological scale: 0, no abnormality; 1, tail atony; 2, hind limb paralysis; 3, paralysis extending to thoracic spine; 4, front limb paralysis; 5, moribund state.

7.2 RESULTS

7.2.1 NEUROPROTECTION BY AUTOIMMUNE anti-MBP T CELLS

Morphological analyses were done to assess the effect of the T cells on the response of the nerve to injury, and specifically on secondary degeneration. Rats were injected intraperitoneally immediately after optic nerve injury with PBS or with 1×10^7 activated T cells of the various cell lines. The degree of primary damage to the optic nerve axons and their attached RGCs was measured by injecting the dye 4-Di-10-Asp

distal to the site of the lesion immediately after the injury. A time lapse of 2 weeks between a moderate crush injury and dye application is optimal for demonstrating the number of still viable labeled neurons as a measure of secondary degeneration, and as the response of secondary degeneration to treatment. Therefore, secondary degeneration was quantified by injecting the dye immediately or 2 weeks after the primary injury, and calculating the additional loss of RGCs between the first and the second injections of the dye. The percentage of RGCs that had survived secondary degeneration was then calculated. The percentage of labeled RGCs (reflecting still-viable neurons) was significantly greater in the retinas of the rats injected with anti-MBP T cells than in the retinas of the PBS-injected control rats (Fig. 2). In contrast, the percentage of labeled 30 RGCs in the retinas of the rats injected with anti-OVA or anti-p277 T cells was not significantly greater than that in the control retinas. Thus, although the three T cell lines accumulated at the site of injury, only the MBP-specific autoimmune T cells had a substantial effect in limiting the extend of secondary degeneration. Labeled RGCs of injured optic nerves of rats injected with PBS (Fig. 3A), with anti-p277 T cells (Fig. 3B) or with anti-MBP T cells (FIG. 3C) were compared morphologically using micrographs.

7.2.2 CLINICAL SEVERITY OF EAE

Animals were injected i.p. with 10^7 T_{MBP} cells with or without concurrent optic nerve crush injury. The clinical course of the rats injected with the T_{MBP} cells was evaluated according to the neurological paralysis scale. Each group contained 5-9 rats. The functional autoimmunity of the injected anti-MBP T cells was demonstrated by the development of transient EAE in the recipients of these cells. As can be seen in Fig. 4A, the course and severity of the EAE was not affected by the presence of the optic nerve crush injury.

7.2.3 SURVIVAL OF RGCS IN NON-INJURED NERVES

Animals were injected i.p. with 10^7 T_{MBP} cells or PBS. Two weeks later, 4-Di-10-Asp was applied to the optic nerves. After five days the retinal were excised and flat

mounted. Labeled RGCs from five fields (located at approximately the same distance from the optic disk), in each retina were counted and their average number per area (mm^2) was calculated.

As can be seen in Fig. 4B, there is no difference in the number of surviving RGCs per area (mm^2) in non-injured optic nerves of rats injected with anti-MBP T cells compared to in rats injected with PBS.

7.2.4. NEUROPROTECTION BY T CELLS REACTIVE TO A CRYPTIC EPITOPE

To determine whether the neuroprotective effect of the anti-MBP T cells is correlated with their virulence, the effect of T cells reactive to a "cryptic" epitope of MBP, the peptide 51-70 (p51-70) was examined. "Cryptic" epitopes activate specific T cells after an animal is immunized with the particular peptide, but not with the whole antigen (Mor et al., *J. Immunol.* 155:3693-3699. 1995). The T cell line reactive to the whole MBP and the T cell line reactive to the cryptic epitope p51-70 were compared for the severity of the EAE they induced, and for their effects on secondary degeneration. In rats injected with the T cell line reactive to the cryptic epitope, disease severity (as manifested by the maximal EAE score) was significantly lower than that in rats injected with the T cell line reactive to the whole protein (Table 1). Whereas anti-MBP T cells caused clinical paralysis of the limbs, rats injected with the anti-p51-70 T cells developed only tail atony, not hind limb paralysis, and almost none showed weakness of the hind limbs. Despite this difference in EAE severity, the neuroprotective effect of the less virulent (anti-p51-70) T cells was similar to that of the more virulent (anti-MBP) T cells (Fig. 5). The percentage of RGCs surviving secondary degeneration in the retinas of rats injected with either of the lines was significantly higher than in the retinas of the PBS-injected rats. Thus, there was no correlation between the neuroprotective effect of the autoimmune T cells and their virulence. It is possible that the anti-p51-70 T cells encounter little antigen in the intact CNS, and therefore cause only mild EAE. Their target antigen

may however become more available after injury, enabling these T cells to exert a neuroprotective effect.

TABLE 1. Anti-MBP and anti-p51-70 T cells Vary in Pathogenicity

<u>T Cell Line</u>	<u>Clinical EAE</u>	<u>Mean Max. Score</u>
Whole MBP	Moderate to severe	2.00 + 0.2
p51-70 of MBP	Mild	0.70 + 0.2

Immediately after optic nerve crush injury, Lewis rats were injected with activated anti-MBP T cells or anti-p51-70 T cells. The clinical course of EAE was evaluated according to the neurological paralysis scale. The mean maximal (max.) score \pm s.e.m. was calculated as the average maximal score of all the diseased rats in each group. The table is a summary of nine experiments. Each group contains five to ten rats. Statistical analysis showed a significant difference between the mean maximal score of rats injected with anti-MBP T cells and that of rats injected with anti-p51-70 T cells ($P=0.039$, Student's t-test).

7.2.5 ELECTROPHYSIOLOGICAL ACTIVITY

To confirm the neuroprotective effect of the anti-MBP T cells, electrophysiological studies were done. Immediately after optic nerve injury, the rats were injected intraperitoneally with PBS or with 1×10^7 activated anti-MBP or anti-OVA T cells. The optic nerves were excised 7, 11 or 14 days later and the compound action potentials (CAPs), a measure of nerve conduction, were recorded from the injured nerves. On day 14, the mean CAP amplitudes of the distal segments recorded from the injured nerves obtained from the PBS-injected control rats were 33% to 50% of those recorded from the rats injected with the anti-MBP T cells (Fig. 6A, Table 2). As the distal segment of the injured nerve contains both neurons that escaped the primary insult and injured neurons that have not yet degenerated, the observed neuroprotective effect could reflect the rescue of spared neurons, or a delay of Wallerian degeneration of the injured neurons (which normally occurs in the distal stump), or both. No effect of the injection anti-MBP T cells on the mean CAP amplitudes of uninjured nerves was

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observed (Fig. 6B, Table 2). It is unlikely that the neuroprotective effect observed on day 14 could have been due to the regrowth of nerve fibers, as the time period was too short for this.

The strong neuroprotective effect of the anti-MBP T cells seen on day 14 was associated with a significantly decreased CAP amplitude recorded on day 7 (Table 2). The anti-MBP T cells manifested no substantial effect on the uninjured nerve on day 7, indicating that the reduction in electrophysiological activity observed in the injured nerve on day 7 might reflect the larger number of T cells present at the injury site relative to the uninjured nerve (Fig. 1). The observed reduction in CAP amplitude in the injured nerve on day 7 reflected a transient resting state in the injured nerve. This transient effect has not only disappeared, but was even reversed by day 14 (Table 2). Early signs of the neuroprotective effect could already be detected on day 11 in the rats injected with anti-OVA T cells, no reduction in CAP amplitude on day 7 could be detected in either the injured or the uninjured nerves, and no neuroprotective effect was observed on day 14 (Table 2). Thus, it seems that the early reduction in CAP and the late neuroprotection shown specifically by the anti-MBP T cells are related.

TABLE 2. Transient reduction in electrophysiological activity of the injured optic nerve induced by anti-MBP T cells, followed by a neuroprotective effect

	<u>Uninjured Optic Nerve</u>	<u>Injured Optic Nerve</u>		
	<u>Day 7</u>	<u>Day 14</u>	<u>Day 7</u>	<u>Day 14</u>
Ratio (%) T _{MBP} /PBS	89.9±9.4 (n=22)	101.2±22.7 (n=10)	63.8*±14.9 (n=17)	243.1**±70.8 (n=8)
Ratio (%) T _{OVA} /PBS	109.7±13.2 (n=11)	92.5±12.6 (n=3)	125.5±24.4 (n=11)	107.3±38.9 (n=4)

Immediately after optic nerve injury, rats were injected with PBS or with activated anti-MBP or anti-OVA T cells. After 7 or 14 days, the CAPs of injured and uninjured nerves were recorded. Ratios were calculated for uninjured nerves as (mean CAP of uninjured nerves from T cell-injected rats/mean CAP of uninjured nerves from PBS-injected rats) x 100, or for injured

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nerves as (mean CAP of injured nerves from T cell-injected rats/mean CAP of injured nerves from PBS-injected rats) x 100. The P value was calculated by comparing the logarithms of the normalized CAP amplitudes of nerves from PBS-injected rats and rats injected with T cells, using the unpaired Student's test, *P<0.05; **P<0.001 n=sample size.

7.3 NEUROPROTECTION IN SPINAL CORD INJURY

7.3.1. MATERIALS AND METHODS

Animals, antigens (MBP, OVA) and T cell lines were as described hereinbefore in 6.1.1, 6.1.3 and 6.1.4, respectively

Contusion. Adult rats (300 to 350g) were anesthetized and the spinal cord was exposed by laminectomy at the level of T7-T8. One hour after induction of anesthesia, a 10 gram rod was dropped onto the laminectomized cord from a height of 50 mm. The impactor device (designed by Prof. Wise Young) allowed, for each animal, measurement of the trajectory of the rod and its contact with the spinal cord to allow uniform lesion. Within an hour of the contusion, rats were injected i.p., on a random basis, with either 10^7 cells (specific to either MBP or OVA, depending on the experimental design) or with PBS. Bladder expression was done at least twice a day (particularly during the first 48h after injury, when it was done 3 times a day) until the end of the second week, by which time the rats had developed autonomous bladder voidance. Approximately twice a week, locomotor activity (of the trunk, tail and hind limbs) in an open field was evaluated by placing the rat for 4 min in the middle of a circular enclosure made of molded plastic with a smooth, non-slip floor (90 cm diameter, 7 cm wall height).

7.3.2 RESULTS

The present study of spinal cord neuroprotection was prompted by the previous example that partial injury to an optic nerve can be ameliorated administering T cells directed to a CNS self-antigen. The question was whether autoimmune T cells could have a beneficial effect on recovery from traumatic spinal cord injury with its greater mass of injured CNS tissue and the attendant spinal shock.

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Adult Lewis rats were subjected to a calibrated spinal cord contusion produced by dropping a 10 gram weight from a height of 50 mm onto the laminectomized cord at the level of T7-T8 (see description included in Basso et al., Exp Neurol 139, 244-256, 1996). The rats were then injected intraperitoneally with autoimmune T cells specific to MBP. Control rats were similarly injured but received either no T cells or T cells specific to the non-self antigen ovalbumin (OVA). Recovery of the rats was assessed every 3 to 4 days in terms of their behavior in an open-field locomotion test, in which scores range from 0 (complete paraplegia) to 21 (normal mobility). The locomotor performance of the rats was judged by observers blinded to the identity of the treatment received by the rats. Included in the study was a group of uninjured, sham-operated (laminectomized but not contused) rats which were injected with anti-MBP T cells to verify the activity of the T cells. In all the sham-operated rats, the anti-MBP T cells induced clinical experimental autoimmune encephalomyelitis (EAE), which developed by day 4, reached a peak at day 7 and resolved spontaneously by day 11. Note, therefore, that at the early post-traumatic stage, any effect of the autoimmune T cells on the injured spinal cord, whether positive or negative, would be transiently masked both by spinal shock and by the paralysis of EAE.

Indeed, none of the rats with contused spinal cords showed any locomotor activity in the first few days after the contusion (Fig. 7A). Interestingly, however, the rats treated with anti-MBP T cells recovered earlier from spinal shock; on day 11, for example, when no recovery could be detected in any of the untreated control rats, significant improvement was noted in the T cell-treated rats (Fig. 7A). At all time points thereafter, the rats that had received the autoimmune T cells showed better locomotor recovery than did the untreated injured rats (Fig. 7A). Thus the autoimmune T cells, in spite of being encephalitogenic, did confer significant neuroprotection. Moreover, the phase of neuroprotective activity coincided with the phase of immune paralysis, supporting our suggestion that neuroprotection might be related to transient paralysis.

By one month after trauma the rats in both groups had reached a maximal behavioral score, which then remained at plateau for at least 3 months of follow-up. In the untreated rats, maximal recovery of locomotor behavior, as noted in previous reports of similarly severe contusion (Basso et al., *supra*), was marked by some ineffectual movement of hind-limb joints, but the rats showed no ability support their body weight and walk, and obtained a score of 7.3 ± 0.8 (mean \pm SEM). In contrast, the average score of the rats that had been treated with the anti-MBP T cells was 10.2 ± 0.8 , and in some rats the value was high as 13. All the rats in the treated group could support their body weight and some could frequently walk in a coordinated fashion. The difference between the two groups, based on 2-factor repeated ANOVA, was statistically significant ($p < 0.05$). The recovery curve based on locomotor activity is nonlinear. The above-described increase in motor activity seen after treatment with the anti-MBP T cells could result from much higher percentage of spared tissue based on a linear regression curve on which the behavioral score is correlated with the amount of neural spinal cord tissue (for example, a difference between 11 and 7) on the locomotion score would be read as a difference between 30% and less than 10% of spared tissue).

In another set of experiments the rats were subjected to a more severe insult, resulting in a functional score of 1.9 ± 0.8 (mean \pm SEM) in the untreated group and 7.7 ± 1.4 in the treated group (Fig. 7B). This difference of more than 3 fold in behavioral scores was manifested by the almost total lack of motor activity in the control rats as compared with the ability of the autoimmune T cell-treated rats to move all their joints. The beneficial effect was specific to treatment with anti-MBP T cells; no effect was observed after treatment with T cells specific to the non-self antigen OVA (data not shown). The positive effect of the autoimmune T cells seems to be expressed in the preservation of CNS tissue that escaped the initial lesion, i.e., in neuroprotection. Therefore, the magnitude of the effect would be inherently limited by the severity of the insult; the more severe the lesion, the less the amount of spared tissue amenable to neuroprotection.

To determine whether clinical recovery could be explained in terms of preservation of spinal axons, we performed retrograde labeling of the descending spinal tracts by applying the dye rhodamine dextran amine (Brandt et al, J-Neurosci-Methods 45:35-40, 1992) at T12, below the site of damage. The number of dye-stained cells that could be counted in the red nucleus of the brain constituted a quantitative measure of the number of intact axons traversing the area of contusion. Sections of red nuclei from injured rats treated with anti-MBP T cells (Fig. 8) contained 5-fold more labeled cells than sections taken from the untreated injured rats. Photomicrographs of red nuclei taken from rats treated with anti-MBP T cells (with an open field score of 10) and from PBS-treated rats (with a score of 6) are shown in Fig. 8. These findings indicate that the reduction in injury-induced functional deficit observed in the T cell-treated rats can be attributed to the sparing of spinal tracts, resulting in a higher degree of neuron viability.

After a follow-up of more than 3 months, when the locomotor activity scores had reached a plateau, the site of injury of three of PBS-treated animals and three animals treated with anti-MBP T cells were analyzed by diffusion-weighted MRI. The cords were excised in one piece from top to bottom and were immediately placed in fixative (4% paraformaldehyde). Axial sections along the excised contused cord were analyzed. Fig. 9 shows the diffusion anisotropy in axial sections along the contused cord of a rat treated with autoimmune T cells, as compared with that of PBS-treated control rat. The images show anisotropy in the white matter surrounding the grey matter in the center of the cord. Sections taken from the lesion sites of PBS-treated control rats show limited areas of anisotropy, which were significantly smaller than those seen at comparable sites in the cords of the rats treated with the anti-MBP T cells. Quantitative analysis of the anisotropy, reflecting the number of spared fibers, is shown in Fig. 9. The imaging results show unequivocally that, as a result of the treatment with the autoimmune anti-MBP T cells, some spinal cord tracts had escaped the degeneration that would otherwise have occurred.

7.3.3 DISCUSSION OF RESULTS

No cure has yet been found for spinal cord lesions, one of the most common yet devastating traumatic injuries in industrial societies. It has been known for more than 40 years that CNS neurons, unlike neurons of the peripheral nervous system, possess only a limited ability to regenerate after injury. During the last two decades, attempts to promote regeneration have yielded approaches that lead to partial recovery. In the last few years it has become apparent that, although most of the traumatic injuries sustained by the human spinal cord are partial, the resulting functional loss is nevertheless far worse than could be accounted for by the severity of the initial insult; the self-propagating process of secondary degeneration appears to be decisive.

A substantial research effort has recently been directed to arresting injury-induced secondary degeneration. All attempts up to now have been pharmacologically based, and some have resulted in improved recovery from spinal shock. The present study, in contrast, describes a cell therapy that augments what seems to be a natural mechanism of self-maintenance and leads, after a single treatment, to long-lasting recovery. The extent of this recovery appears to exceed that reported using pharmacological methods.

In most tissues, injury-induced damage triggers a cellular immune response that acts to protect the tissue and preserve its homeostasis. This response has been attributed to macrophages and other cells comprising the innate arm of the immune system. Lymphocytes, which are responsible for adaptive immunity, have not been thought to participate in tissue maintenance. Adaptive immunity, according to traditional teaching, is directed against foreign dangers. Our studies now show, however, that the adaptive T cell immune response can be protective even when there is no invasion by foreign pathogens. In the case of tissue maintenance, the specificity of the T cells is to tissue self-antigens.

Our observation of post-traumatic CNS maintenance by autoimmune T cells suggests that we might do well to reevaluate some basic concepts of autoimmunity. T cells that are specific

to CNS self antigens in general, and to MBP in particular, have long been considered to be only detrimental to health. In the present study, however, the same T cell preparation that can produce EAE in the undamaged CNS was found to be neuroprotective in the damaged spinal cord, suggesting that the context of the tissue plays an important part in determining the outcome of its interaction with T cells. It would seem that the tissue deploys specific signals to elicit particular T cell behaviors. Among such signals are costimulatory molecules, particularly members of the B7 family (Lenchow et al., Annu. Rev. Immunol. 14:233-258, 1996). As shown hereinafter, the injured rat optic nerve transiently expresses elevated levels of the costimulatory molecule B7.2, which is constitutively expressed at low levels in the rat CNS white matter and which is thought to be associated with regulation of the cytokine profile of the responding T cells (H. L. Weiner, Annu. Rev. Med. 48:341-51, 1997). The early post-injury availability of the exogenous anti-MBP T cells, coinciding with the observed early post-injury increase in B7.2 would support the idea that signals expressed by the tissue might modulate the T cell response. It is thus conceivable that anti-MBP T cells which cause a monophasic autoimmune disease upon interacting with a healthy CNS nerve, might implement a maintenance program when they interact with damaged CNS tissue expressing increased amounts of B7.2 and probably other costimulatory molecules. The neuroprotective effects of the T cells may be mediated, at least in part, by antigen-dependent regulation of specific cytokines or neurotrophic factors (M. Kerschensteiner et al., J. Exp. Med. 189:865-870, 1999) produced locally at the site of injury.

Thus, the present invention is also directed to manipulating B7.2 co-stimulatory molecule to prevent or inhibit neuronal degeneration and ameliorate the effects of injury to or disease of the nervous system. B7.2 molecule can be up-regulated for this purpose, using drugs or by genetic manipulation, without undue experimentation.

In a recent study, it was reported that injury to the spinal cord triggers a transient autoimmune response to MBP (Popovich et al., J. Neurosci. Res. 45:349-63, 1996). However,

whether that response is detrimental or beneficial remained an open question (Popovich et al, *J. Comp. Neurol.* 377:443-464, 1997). From our present data, it would appear that the activation of anti-MBP T cells could indeed be beneficial. However, a supplement of exogenous autoimmune T cells may be required to overcome the restrictions on immune reactivity imposed by the immune-privilege of the CNS (J. W. Streilein, *Science* 270:1158-1159, 1995). The finding that autoimmune response can be advantageous suggests that natural autoimmune T cells may have undergone positive selection during ontogeny, as proposed by the theory of the immunological homunculus (I. R. Cohen, *Immunol. Today* 13, 490-494 (1992), and are not merely a default resulting from the escape from negative selection of T cells that recognize self antigens (C. A. Janeway, Jr., *Immunol. Today* 13:11-6, 1992). Such a response could then be considered as a mechanism of potential physiological CNS self-maintenance, which is, however, not sufficient for the purpose because of the immune-privileged character of the CNS.

A single injection of autoimmune T cells lasted for at least 100 days. Thus, this procedure offers a form of self-maintenance. This specific autoimmune response, when properly controlled, is useful as part of a self-derived remedy for spinal cord injury.

EXAMPLE: NEUROPROTECTIVE EFFECTS OF NS-SPECIFIC ANTIGEN

8.1 MATERIALS AND METHODS

Animals, crush injury of rat optic nerve, and retrograde labeling are described above in Sections 6 and 7. A peptide based on amino acids 35-55 of myelin/oligodendrocyte glycoprotein (MOG p35-55) was chemically synthesized at the Weizmann Institute, Israel.

8.1.1 INHIBITION OF SECONDARY DEGENERATION

Rats were injected intradermally in the footpads with MOG p35-55 (50 µg/animal) and IFA, or PBS ten days prior to optic nerve crush injury. Retinal ganglion cells were assessed two weeks after injury using retrograde labeling as described above. The number of RGCs in rats injected with PBS or MOG

p35-55 was expressed as a percentage of the total number of neurons in rats injected with MOG p35-55 in the absence of crush injury.

8.2 RESULTS

As shown in Fig. 10, the number of labeled retinal ganglion cells (indicating viable axons) was about 12.5 fold greater in animals injected with MOG p35-55 compared to animals receiving PBS.

EXAMPLE: NEUROPROTECTIVE EFFECTS OF MBP ADMINISTERED ORALLY

9.1 MATERIALS AND METHODS

Animals, crush injury of rat optic nerve, and retrograde labeling of RGCs are described above in Sections 6 and 7.

9.1.1 INHIBITION OF SECONDARY DEGENERATION

Bovine MBP (Sigma, Israel) (1 mg/dose) was administered to rats by gavage using a blunt needle. MBP was administered 5 times, every third day, beginning 2 weeks prior to optic nerve crush injury. The number of RGCs in treated animals was expressed as a percentage of the total number of neurons in animals subjected to optic nerve crush injury but which did not receive MBP.

9.2 RESULTS

As shown in Fig. 11, the number of labeled RGCs was about 1.3 fold greater in animals treated with MBP compared to untreated animals.

9.3 THE B7.2 COSTIMULATORY MOLECULE IS ASSOCIATED WITH POST-TRAUMATIC MAINTENANCE OF THE OPTIC NERVE BY ORAL ADMINISTRATION OF MBP

9.3.1 INTRODUCTION

Autoimmune T cells can under certain conditions be beneficial to traumatized CNS axons. The effect of such T cells on the damaged tissue might be influenced by the nature

and amount of the costimulatory molecules it expresses. We show that the B7.2 costimulatory molecule is constitutively expressed in the intact rat optic nerve, and after injury is up-regulated at the margins of the injury site. Pre-injury induction of oral tolerance to MBP resulted in a further post-injury increase in B7.2 at the margins and at the injury site itself, as well as a better preservation of the traumatized nerve. Thus, B7.2 expression in the brain and its up-regulated after trauma seem to be directly related to post-traumatic maintenance displayed by autoimmune T cells.

Neuronal injury in the CNS causes degeneration of directly damaged fibers as well as of fibers that escaped the primary insult. It also triggers a systemic response of autoimmune T cells to MBP, that might affect the course of degeneration of the injured nerve. Whether the effect of these T cells on the nerve is detrimental or beneficial may depend, in part, on the nature and level of the costimulatory molecules expressed by the damaged tissue. Several costimulatory molecules have recently been identified, including the B7 and CD40 molecules (Caux et al., "Activation of Human Dendritic Cells Through CD40 Cross-Linking", *J. Exp. Med.* 180:1263-1272, 1994; and Lenschow et al., "CD28/B7 System of T Cell Costimulation", *Annu. Rev. Immunol.* 14:233-258, 1996). CD40 appears to be dominant during cell differentiation in the lymph nodes and B7 during activation of T cells in the target organ (Grewal et al., "Requirement for CD40 Ligand in Costimulation Induction, T Cell Activation, and Experimental Allergic Encephalomyelitis", *Science* 273:1864-1867, 1996). B7 costimulatory molecules are expressed on antigen-presenting cells (APCs) as B7.1 or B7.2., which might preferentially support activation of the Th1 or the Th2 type of immune response, respectively (Kuchroo et al., "B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy", *Cell* 80:707-718, 1995; and Karandikar et al., "Targeting the B7/CD28:CTLA-4 costimulatory system in CNS autoimmune disease", *J. Neuroimmunol.* 89:10-18, 1998). We were therefor interested in determining the identity B7 subtype expressed in intact and injured CNS white matter, and its

possible influence on the course of the response to the injury.

9.3.2 RESULTS

The costimulatory molecule expressed constitutively in the intact optic nerves of adult Lewis rats was identified as B7.2. (Figs. 12A, 12B). To examine the effects of neurotrauma on the expression of B7 costimulatory molecules, we inflicted a mild crush injury on the optic nerves of Lewis rats and assessed the neural expression of B7 by immunohistochemical analysis. The most striking effect of the injury was seen on B7.2 expression manifested on post-injury day 3 by its elevation at the margins of the injury site (Figs. 12C,D,E). In contrast, expression of B7.1 was not detected in the optic nerve either before or 3 days after injury. On day 7, however, B7.1 was detectable at the site of injury, having pattern reminiscent of that seen for macrophages or microglia (Fig. 12F).

Next, we attempted to determine whether the degenerative response to optic nerve injury could be modified by peripheral manipulation of the immune system. The manipulation chosen was induction of oral tolerance, known to cause a "bystander" T cell immunosuppressive effect (Weiner et al., "Tolerance Immune Mechanisms and Treatment of Autoimmune Diseases", Immunol. Today 18:335-343, 1997). Ingestion of low doses of MBP results in the activation of T cells which, based on antigen recognition, secrete TGF as the dominant cytokine and thus favor an immune response of Th2/3 type (Chen, Y., "Regulatory T Cell Clones Induced by Oral Tolerance: Suppression of Autoimmune Encephalomyelitis", Science 265: 1237-1240, 1994).

Lewis rats were fed with food to which 1 mg of bovine MBP had been added five times daily every other day. Ten days after first receiving the supplement, the rats were subjected to mild unilateral optic nerve crush injury. This time interval between initiation of oral tolerance and injury was chosen to allow adequate build-up of the systemic T cell response. As shown in Fig. 13A and B, the numbers of macrophages or active microglia (indicated by ED-1 labeling)

and T cells (indicated by immunolabeling for T cell receptor), assessed 3 days after injury, did not differ from those observed in control injured rats which did receive any treatment or were fed with PBS. In the rats with induced oral tolerance to MBP, however, the amounts B7.2 were further increased at the margins of the site of injury (Fig. 13C) as compared with controls (Fig. 12E). In addition, B7.2 in the rats with induced oral tolerance to MBP was also elevated at the site of injury relative to the control nerves (Fig. 13C). It seems reasonable to assume that the T cells exposed to MBP via intestinal absorption, upon invading the injured CNS, contributed to the increase in expression of B7.2 by the injured nerve.

We then attempted to determine whether the observed changes in B7.2 expression in the injured rats was correlated with the extent of neuronal degeneration. Acute injury of the rat optic nerve is followed by a process of nerve degeneration, which can be quantified by retrograde labeling of the surviving neurons and counting of the corresponding cell bodies. Two weeks after optic nerve injury the number of surviving retinal ganglion cells (RGCs), representing still-viable neurons, in the group of MBP-fed rats was significantly higher than that in the control group, or than in the group of rats with injured nerves that were fed with ovalbumin. Interestingly, the benefit of the induced oral tolerance to MBP was increased by feeding the rats with more intensive schedule (Fig. 14).

DISCUSSION OF EXPERIMENTAL RESULTS

The results of the experiments described in Sections 6 and 7 show that activated T cells accumulate at a site of injury in the CNS. Furthermore, the results also demonstrate that the accumulation of T cells at the site of injury is a non-specific process, i.e., T cells which accumulated at the site of injury included both T cells which are activated by exposure to an antigen present at the site of injury as well as T cells which are activated by an antigen not normally present in the individual.

The results of experiments described in Section 7 demonstrate that the beneficial effects of T cells in

ameliorating damage due to injury in the CNS are associated with an NS-specific self-antigen as illustrated by MBP. More specifically, the administration of non-recombinant T cells which were activated by exposure to an antigen which can cause autoimmune disease (T_{MBP}), rather than aggravating the injury, led to a significant degree of protection from secondary degeneration. Thus, activating T cells by exposure to a fragment of an NS-specific antigen was beneficial in limiting the spread of injury in the CNS. The present findings show that secondary degeneration can be inhibited by the transfer into the individual on non-recombinant T cells which recognize an NS-specific self antigen which is present at a site of injury. The T cells may recognize cryptic or non-pathogenic epitopes of NS-self antigens.

In addition, the studies described in Sections and 9 show that activation of T cells by administering an immunogenic antigen (e.g. MBP) or immunogenic epitope of an antigen (e.g. MOG p35-55), may be used for preventing or inhibiting secondary CNS degeneration following injury.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method step, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to

the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same function can be used; and it is intended that such expressions be given their broadest interpretation.

All publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A composition for preventing or inhibiting degeneration in the central nervous system or peripheral nervous system for ameliorating the effects injury or disease, comprising:

- (a) NS-specific activated T cells;
- (b) NS-specific antigen;
- (c) a peptide derived from an NS-specific antigen;
- (d) a nucleotide sequence encoding an NS-specific antigen;
- (e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or
- (f) any combination of (a)-(e).

2. A composition according to claim 1, for promoting nerve regeneration in the central nervous system or peripheral nervous system for ameliorating the effects of injury or disease.

3. The composition of claim 1 or 2 in which said injury comprises spinal cord injury, blunt trauma, penetrating trauma, hemorrhagic stroke, or ischemic stroke.

4. The composition of claim 1 or 2 in which said disease is Diabetic neuropathy, senile dementia, Alzheimer's disease, Parkinson's Disease, facial nerve (Bell's) palsy, glaucoma, Huntington's chorea, amyotrophic lateral sclerosis, non-arteritic optic neuropathy, or vitamin deficiency.

5. The composition of claim 1 or 2 in which said disease is not an autoimmune disease or a neoplasm.

6. The composition according to any of claims 1-5 wherein said NS-specific activate T cells of (a) are autologous T cells, or allogeneic T cells from related donors, OR HLA-matched or partially matched, semi-allogeneic or fully allogeneic donors.

7. The composition according to claim 6 wherein said autologous T cells have been stored or are derived from autologous CNS cells.

8. The composition according to claim 6 wherein said T cells are semi-allogeneic T cells.

9. The composition according to any of claims 1-5 wherein said NS-specific antigen of (b) is elected from myelin basic protein (MBP), myelin oligodenrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), S-100, β -amyloid, Thy-1, P0, P2 and neurotransmitter receptors.

10. The composition according to any one of claims 1-5 wherein said peptide derived from an NS-specific antigen is an immunogenic epitope or a cryptic epitope of said antigen.

11. The composition according to claim 10 wherein said peptide is an immunogenic epitope or a cryptic epitope derived from MBP.

12. The composition according to claim 11 wherein said peptide corresponds to the sequences p11, p51-70, p91-110, p131-150, or p151-170 of MBP.

13. The compositions according to any one of claims 1-5 and 11-12 in which said NS-specific antigen or a peptide derived therefrom is administered intravenously, orally, intranasally, intrathecally, intramuscularly, intradermally, topically, subcutaneously, mucosally (e.g., orally, intranasally, vaginally, rectally) or buccally.

14. The composition according to claim 13 comprising MBP for oral administration.

15. Use of:

- (a) NS-specific activated T cells;
- (b) an NS-specific antigen;
- (c) a peptide derived from an NS-specific antigen;
- (d) a nucleotide sequence encoding an NS-specific antigen;
- (e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or

(f) any combination of (a)-(e),
for the preparation of a composition for preventing or
inhibiting neuronal degeneration in the central nervous system
or peripheral nervous system for ameliorating the effects of
injury or disease.

16. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral

nervous system, which comprises administering to an individual in need thereof an effective amount of:

- (a) NS-specific activated T cells;
- (b) NS-specific antigen;
- (c) a peptide derived from an NS-specific antigen;
- (d) a nucleotide sequence encoding an NS-specific antigen;
- (e) a nucleotide sequence encoding a peptide derived from an NS-specific antigen, or
- (f) any combination of (a)-(e).

17. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral nervous system comprising administering to an individual in need thereof an effective amount of a composition according to any one of claims 1-13 and actively immunizing said individual to build up a critical T cell response.

18. A method for preventing or inhibiting neuronal degeneration in the central nervous system or peripheral nervous system comprising administering to an individual in need thereof an effective amount of a composition for up-regulating B7.2 costimulatory molecule or genetically manipulating B7.2 costimulatory molecule in said individual.

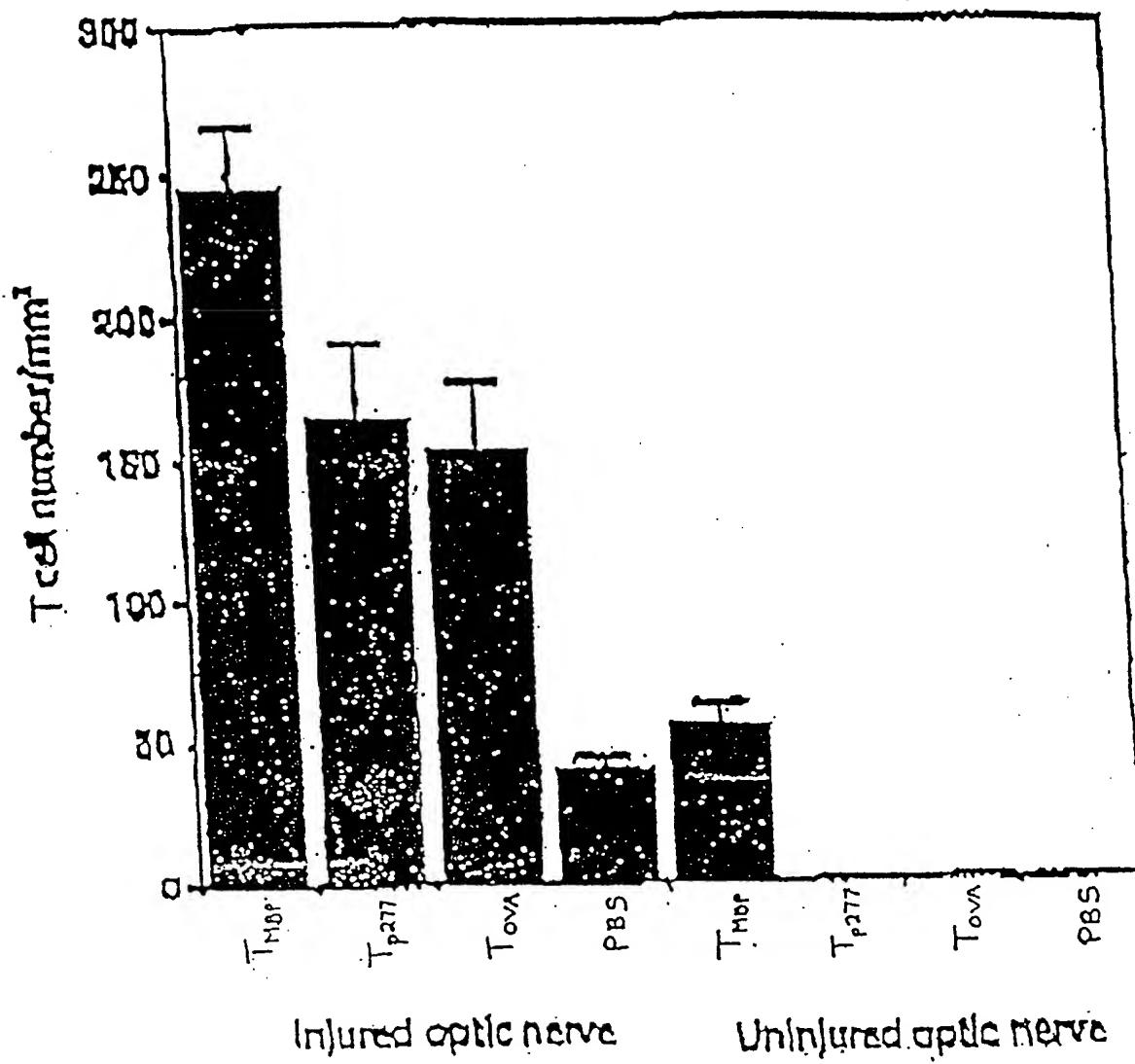
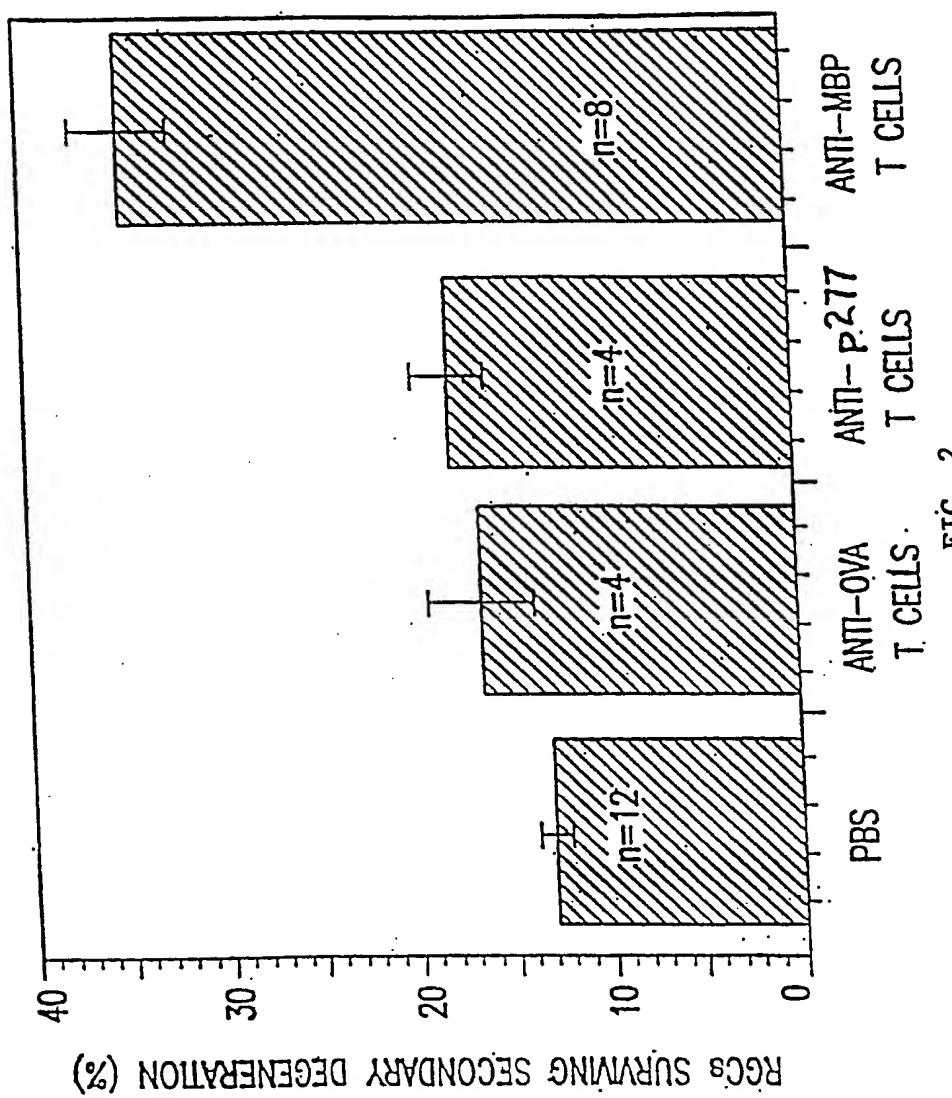


FIG. 1





160 μm

FIG. 3C



FIG. 3B



FIG. 3A

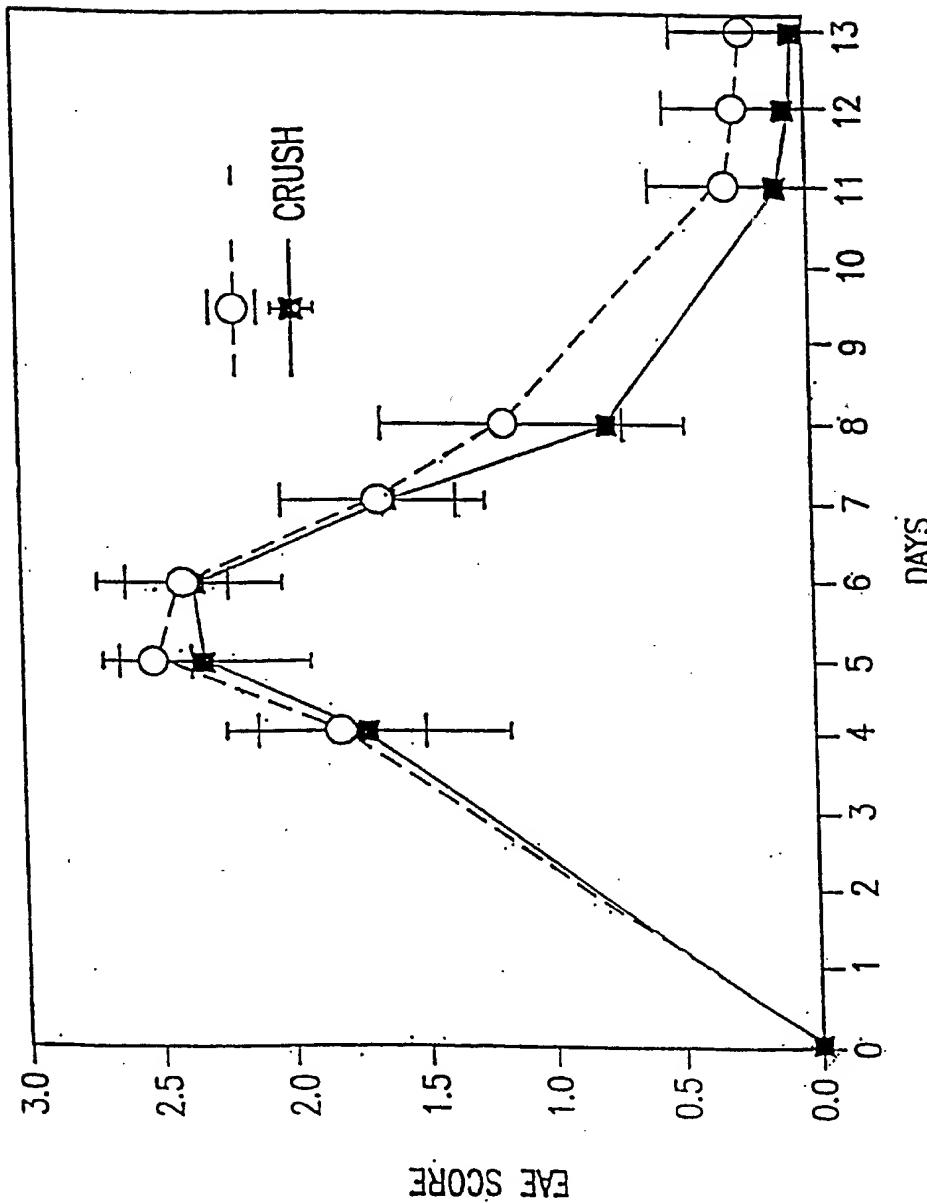


FIG. 4A

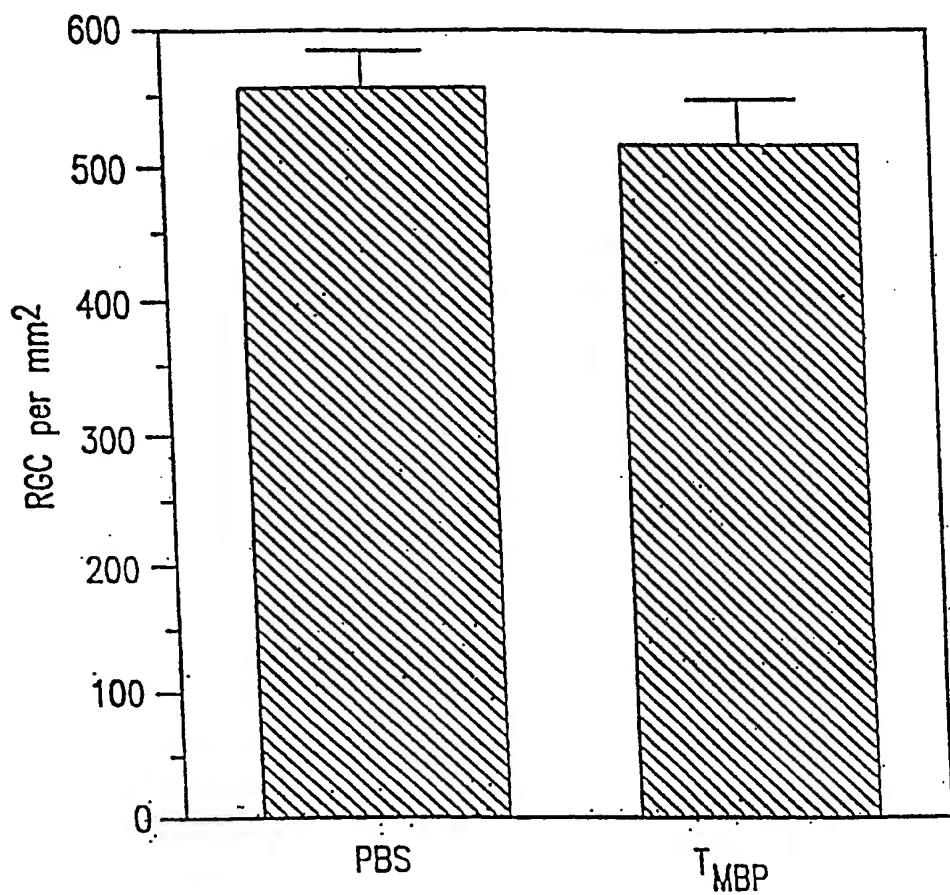


FIG. 4B

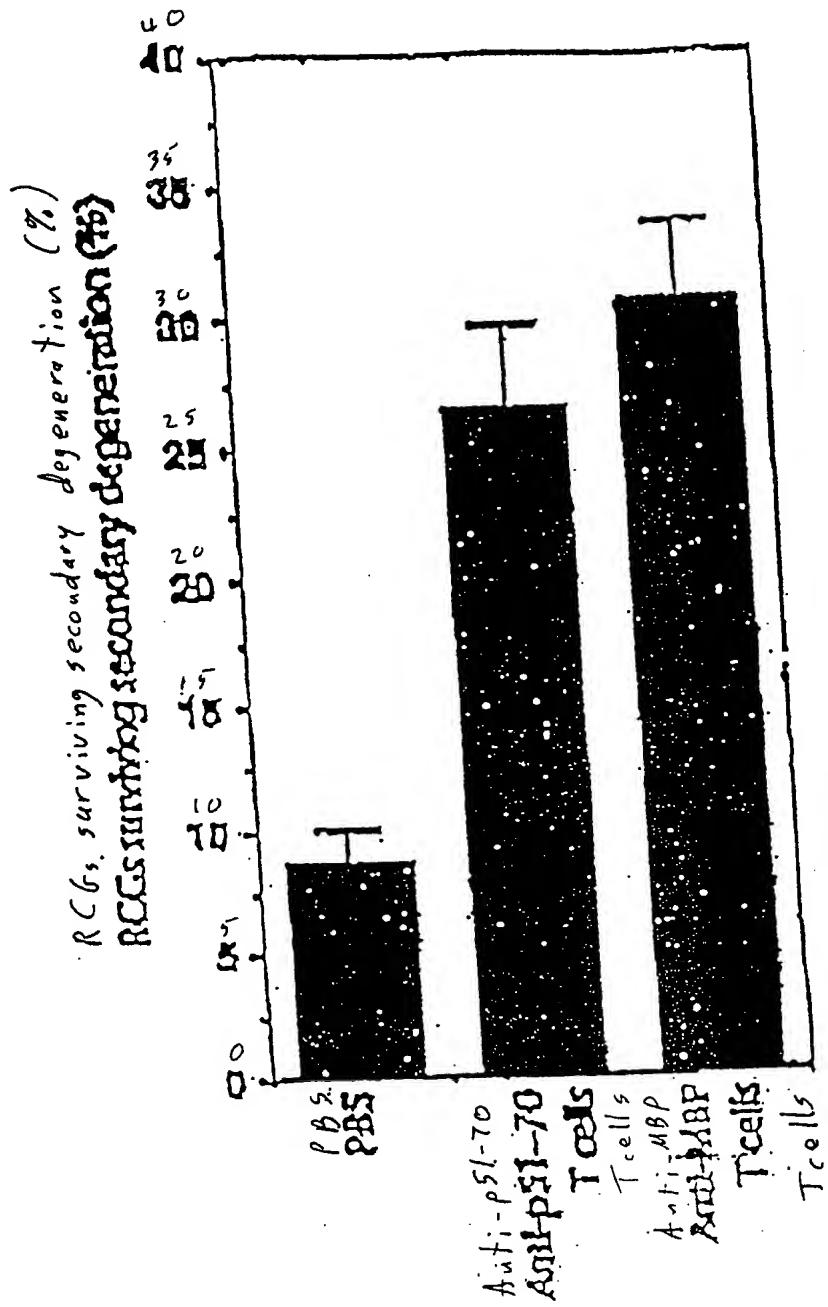


FIG. 5

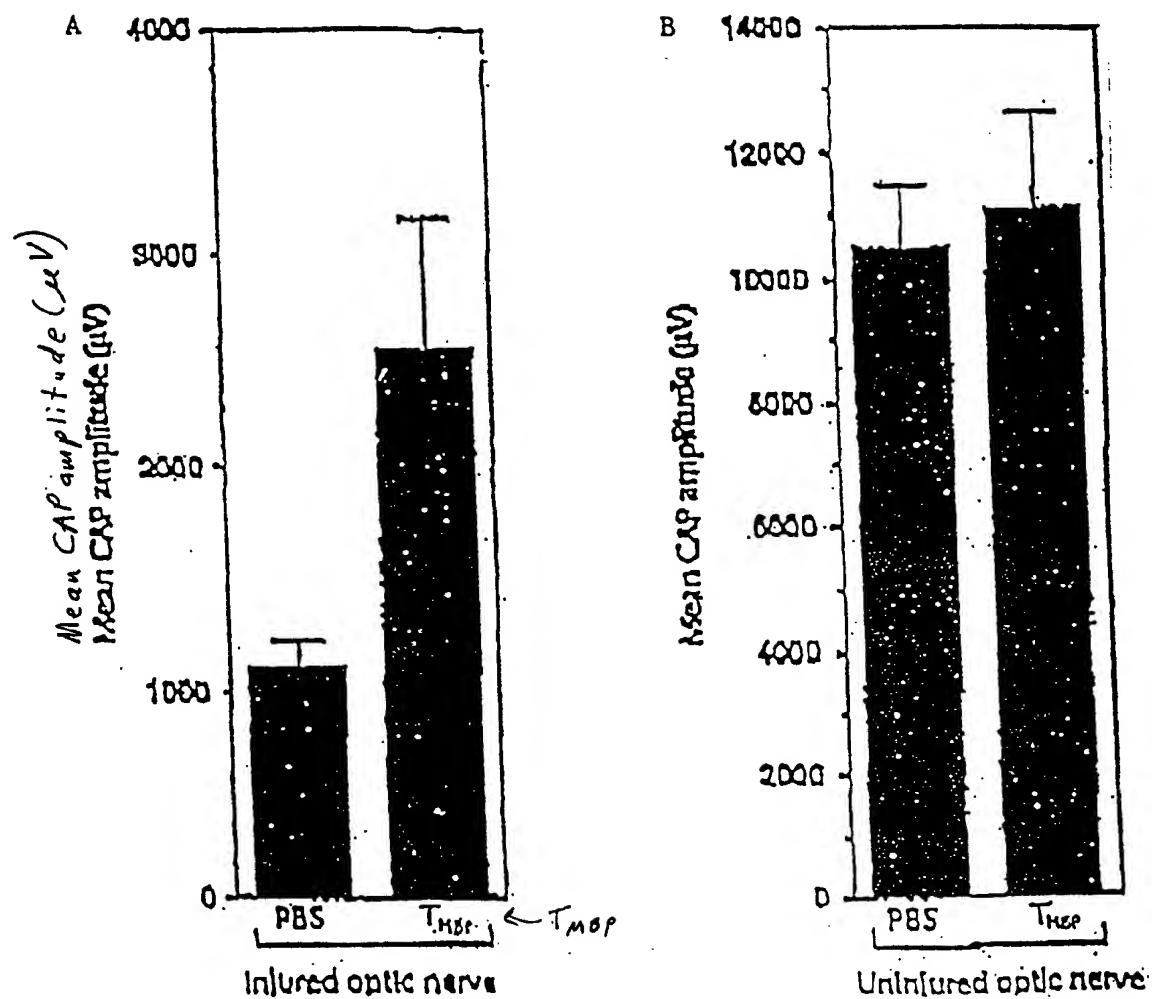


FIG. 6

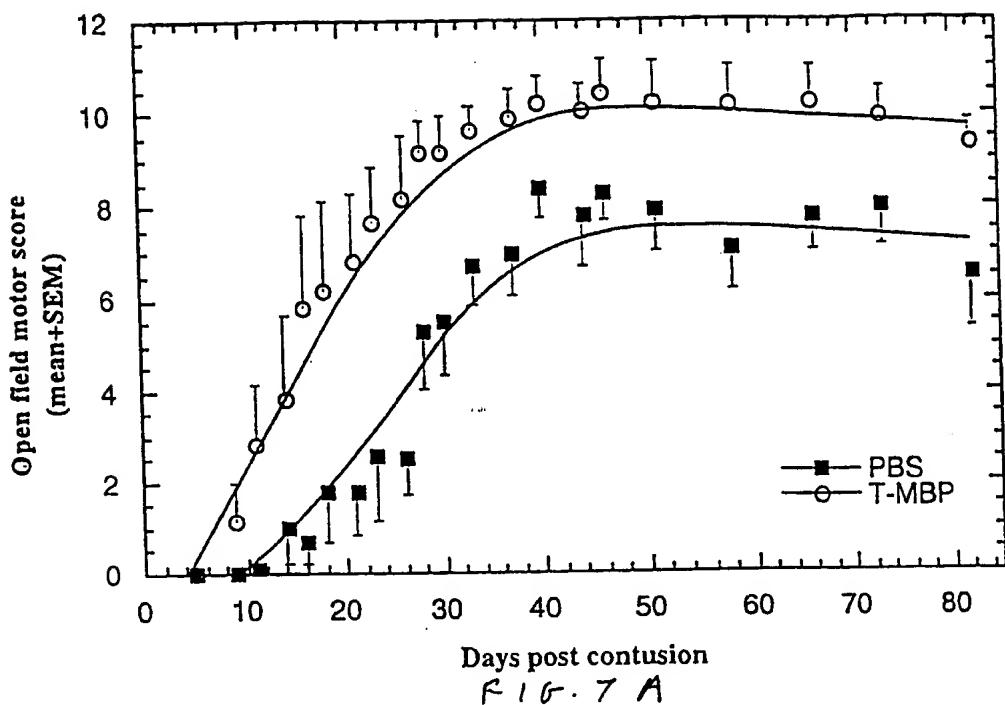


FIG. 7 A

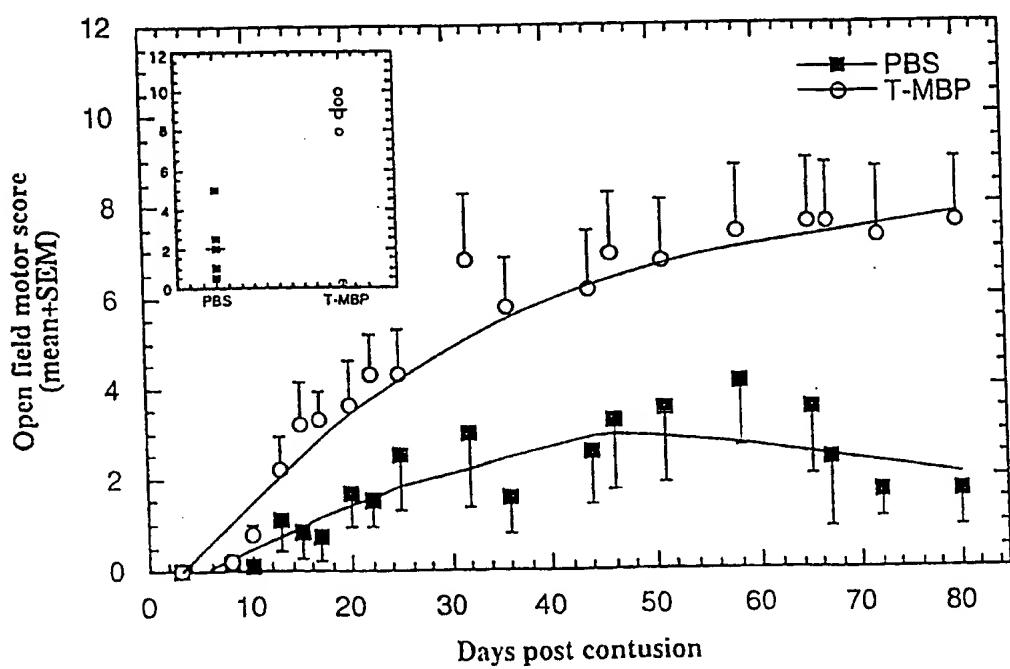


FIG. 7 B

FIG. 8

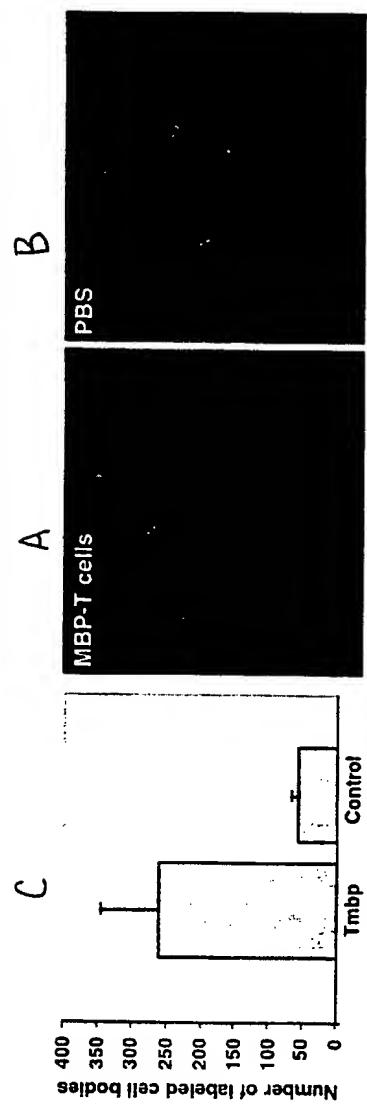
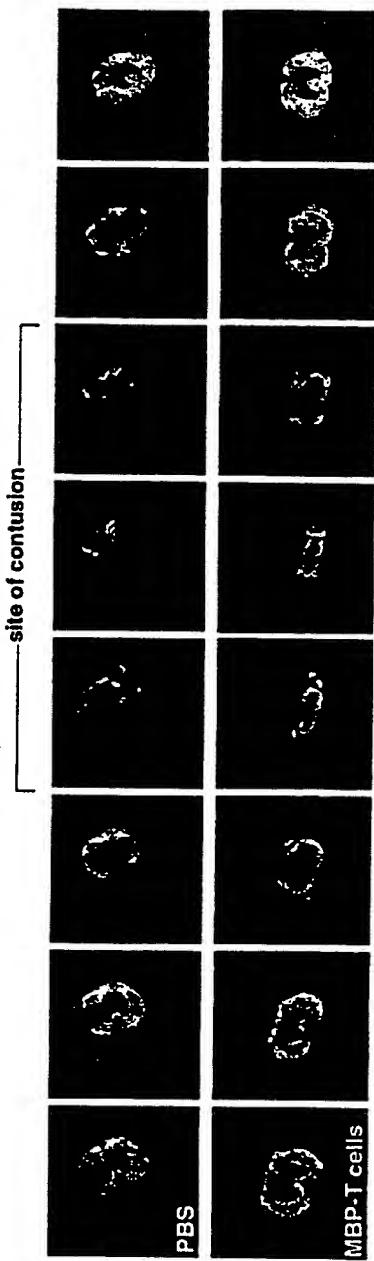


FIG. 9



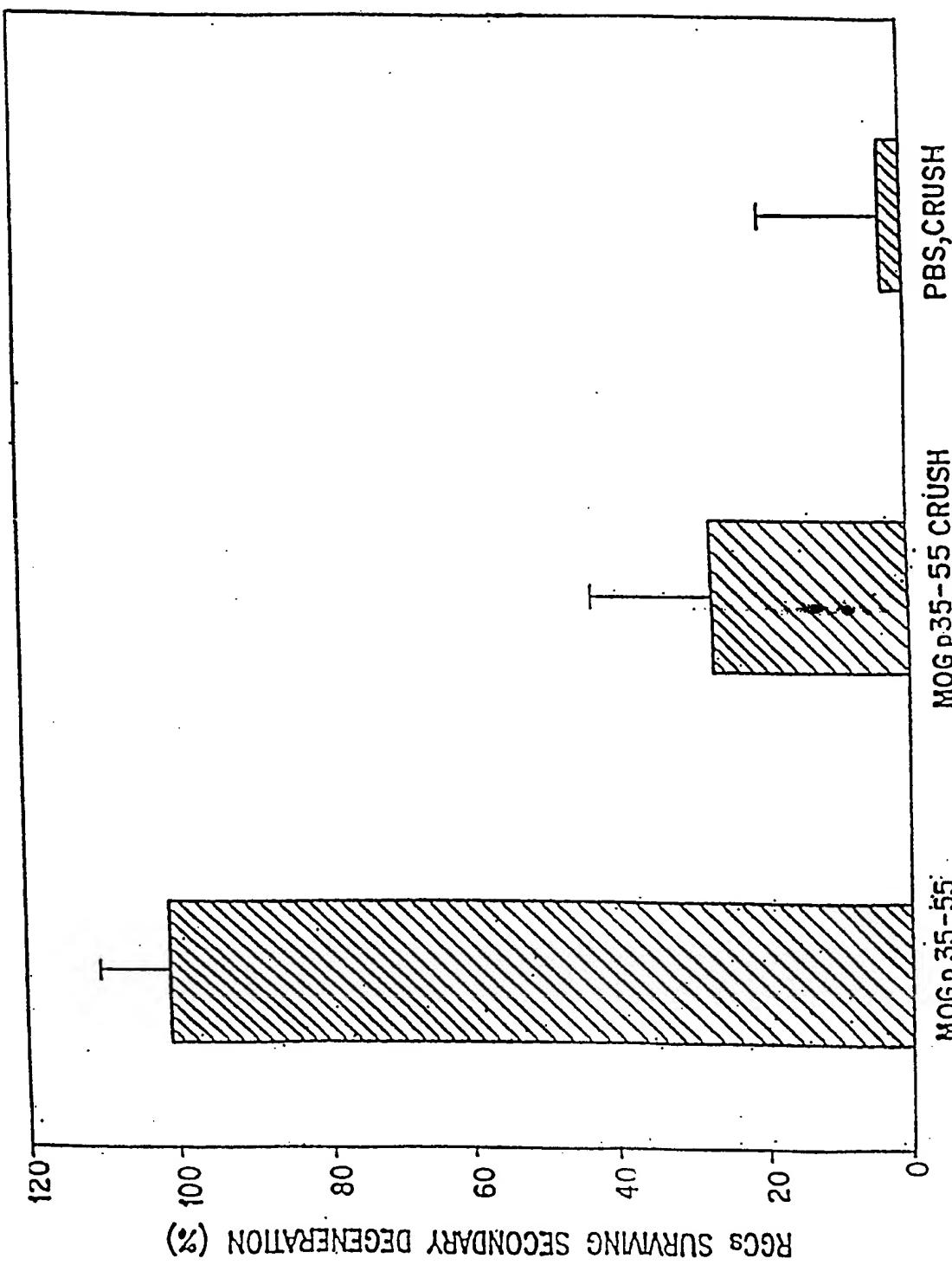


FIG. 10

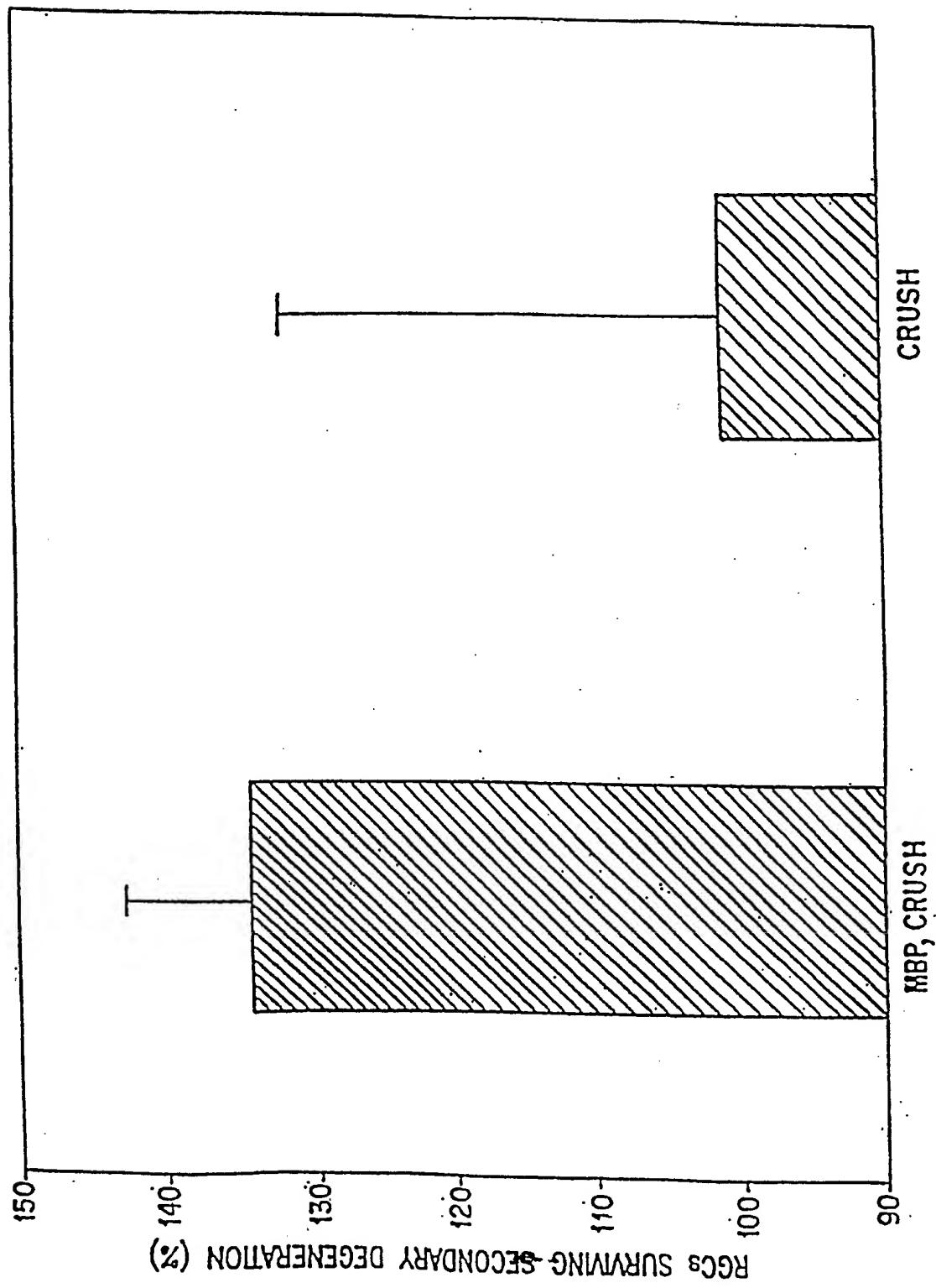


FIG. #1

FIG. 12

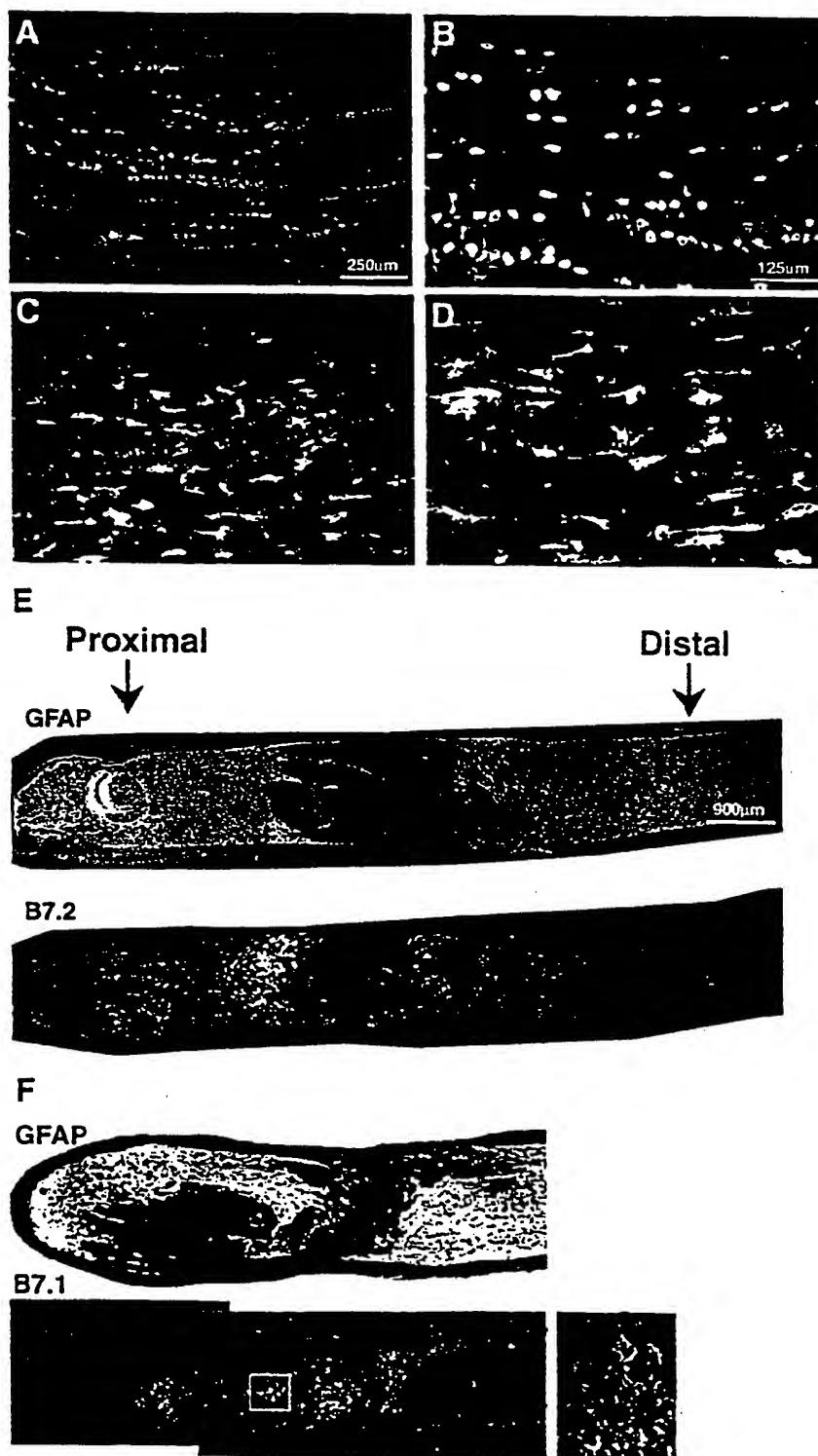


FIG. 13

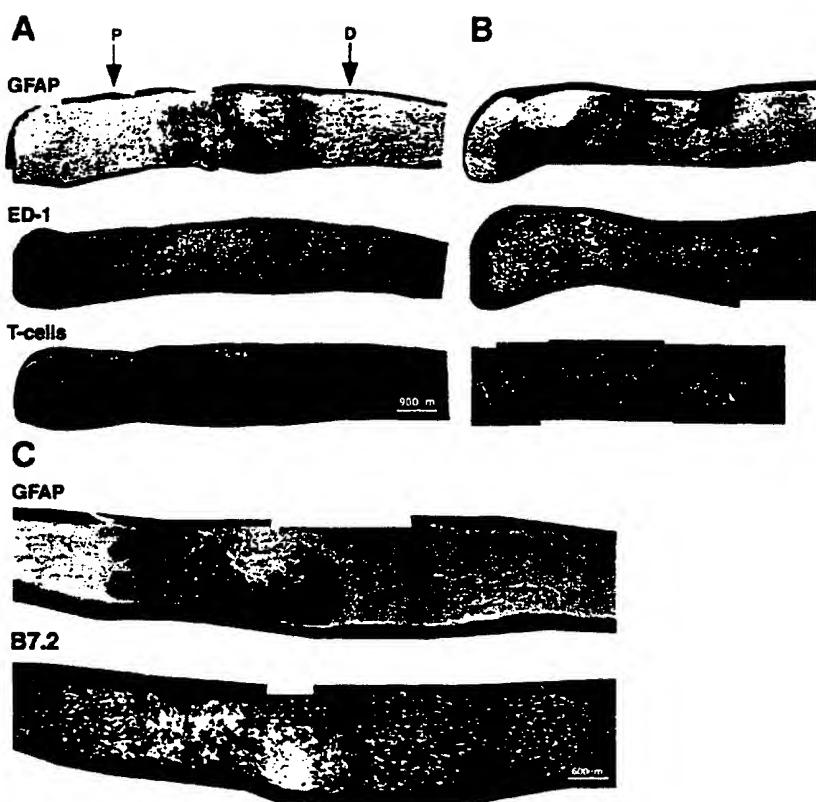
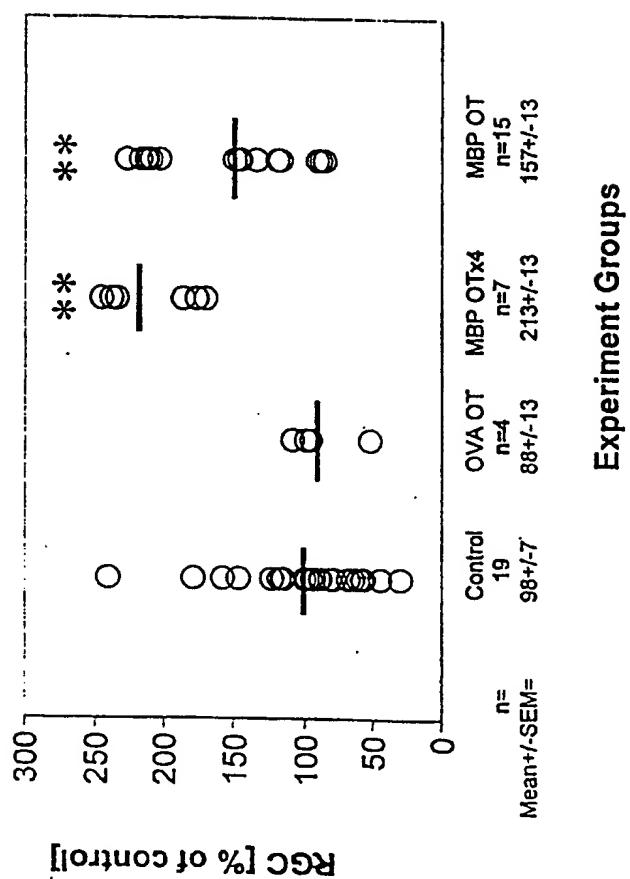


FIG. 14



1 ccaagaagat cccacacgcg cttccgaagg cctggatgtg atggcatcac agaagagacc
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121 tggcttcctc ccaaggcaca gagacacacggg catccttgac tccatcgccg gtttttag
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241 ctacggctcc ctgccccaga agtcgcagag gacccaaat gaaaacccag tagtccactt
301 cttcaagaac attgtgacac ctcgtacacc ccctccatcc caaggaaagg ggagaggct
361 gtccctcagc agatttagct ggggagggaaag agacagccgc tctggatctc ccatggcaag
421 acgctgagag ctcctctgtc cagccttccc qaatcctgcc ctggcttct taatataact
481 gccttaaacg tttaattcta ctgcaccaa atagctagtt agacgagacc ctctcttaat
541 cccgtggggc tgtgaacgcg gcggggccagc ccacggcacc ctgactggct aaaactgttt
601 gtccctttt at

FIG. 15

1 gaaaacagt cagccacctc cgagagcctg gatgtatgg cgtcacagaa gagaccctcc
61 cagaggcacg gatccaagta cctggccaca gcaagtaeca tggaccatgc caggcatggc
121 ttccctccaa ggcacagaga cacgggcate cttgactcca tcgggcgett ctttggcggt
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241 cactatggct ccctgccccca gaagtacac acaccacccc cgtcgtaggg aaaggggaga
301 cacttcttca agaacatgt gacgcctcgc acaccacccc cgtcgtaggg aaaggggaga
361 ggactgtccc tgagcagatt tagctggggg gccgaaggcc agagaccagg atttggctac
421 ggagggcagag cgtccgacta taaatcggtc cacaagggt tcaagggagt cgatgcccag
481 ggcacgctt caaaaatttt taagctggga ggaagagata gtcgctctgg atcacccatg
541 gctagacgct gaaaaccac ctgttccgg aatcctgtcc tcagettctt aatataactg
601 ccttaaaact ttaatcccac ttggccctgt tacctaatta gagcagatga cccctccct
661 aatgcctgcg gagtttgca cgttagtaggg tcagggccacg gcagccattacc ggcaatttcc
721 ggccaacagt taaatgagaa cataaaaaca gaaaacggtt aaaactgtcc ctttctgtgt
781 gaagatcagc ttccttccccca cgcaatgtgc ccccaagacgc acgtgggtct tcagggggcc
841 aggtgcacag acgtccctcc acgttccaccc ctccaccctt ggactttctt ttccgttgg
901 ctcggcaccc ttgcgtttt gctggtaact gccatggagg cacacagctg cagagacaga
961 gaggacgtgg gggcagaga ggactgttg catccaagct tcctttgttt tttttccctg
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1201 cccaaatttc tgaattcgcg tggcgtggag ggaggaaaag gaggcaaacg tgggctgggc
1261 aatggcctca catagggaaac agggtcttcc tggagatttg gtgatggaga tgtcaagcag
1321 gtggcctctg gacgtcaccg ttggccctgca tggggcccc agagcagcct ctatgaacaa
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1561 gataaccggc tcattcaattt cctcccagaa gacgcgtggt agcgagtagg cacaggctg
1621 cacctgctcc cgaattactc accgagacac acgggcttag cagacggccc ctgtgatgga
1681 gacaaagacg tcttctgacc atatccttct taacccgc tggcatctcc tttcgccct
1741 ccctccctaa cctactgacc caccttttga ttttagcga cctgtgttgg ataggccctc
1801 caaagagtcc cacgctggca tcaaccctccc cgaggacgga gatgaggagt agtcagcgtg
1861 atgccaaaac gcgtttctt aatccaattt taattctgaa tggctgtgtt gggcttaata
1921 ccatgtctat taatatatacg cctcgatgtt gagagagttt caaagaacaa aactccagac
1981 acaaaacctcc aaattttca gcaagaagcac tctgcgtcgc ttagctgagg tggcgtctgc
2041 gatccatacg tggccgcacc cacacagcac gtgctgtgac gatggctgaa cgaaaagtgt
2101 acactgttcc tgaatattga aataaaaaca taaactttt

FIG. 16

A. 1 taatatctag ggktttgact ctgaccgtg ttggggctct cacttcatgg cttctcacgc
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 121 ttcattttat ttccacccctc aatccacatt tccagatgtc tctgcagcaa agcgaattc
 181 caqgcaagcc tttagggaaaa aagaaaaaac aaagaaaatg aaacaattgg cagtgaagg
 241 cagaaagaga agatggagcc cttagagaag ggagtatccc tgagtaggtg gggaaaagg
 301 gaggagaagg ggaggaggag aggaggagga aagcaggcct gtcccttaa qggggttggc
 361 tgtcaatcag aaagccctt tcattgcagg agaagaggac aaagatactc agagagaaaa
 421 agtaaaagac cgaagaagga ggctggagag accaggatcc ttccagctga acaaagttag
 481 ccacaaagca gactagccag ccgctacaa ttggagtcag agtcccaaag acatggtaa
 541 gttcaaaaa ctttagattt gaagattcaa gaggacacag g

B. 1 ctgctttcag agcctgtgac ttcttgcgtg cctctccgt ttctcagcaa catggcatag
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 181 ataaagtctg ttttagtgc ttttagaca atctcagct ataactgagg gtggggacat
 241 tagccaagct gcattatagg aggacaaaac tgccatataa agtgtccaaa atattaagc
 301 ctgcattttt attattggaa gtaatataa acctccattt ttccaattttt cattttttt
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 961 ctcttactgc ccaggtgtt gtaaatggc gcaatctgg ctacggcaa cctccgcctc
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 1561 gaaaagctaa ttgagaccta ttctccaa aactaccaag actatgatgta tctcatcaat
 1621 gtgttaagtac ctgcctccccc acacagaccc atctttttt tccctcttc cactctggag
 1681 atagagaact cttcagttacc tttagtaacta gcagggact ggggtggagc cagaccggat
 1741 tccccgactt tccctctgtt ca

FIGS. A-B

C. 1 ctagaaaatc cctagccttg ttaagggtgct cgctctggtg tataacctac ttatgtcggg
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 661 ttttgtatc ctttgcgttcc cttcttagta ggtaccacaa gagtggatc aactggacaa
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 781 ataataaaaa ttaataata actccaaggt aaatttacaa tttcttgg

D. 1 gatcctccctc attttcccc tacccatcc cccccacccctc cgttataactg gggccagttt
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 301 gtgcctgtgt acattttactt caacacctgg accacccgtcc agtctattgc ctccccagc
 361 aagacctctg ccagtatagg cagtcctgt gctgtatgca gaatgtatgg tgagtttaggg
 421 tacgggtgt ttggctctcc taccctactt ggaagcacta tatattttgtt tattttctt
 481 gtgttaaggag ggttgtgatt atgagaaaaa tataagatga tgaatgattt ggtcttagtt
 541 tattaatctt tccctactga aaccagagag gtttcttccc ccggaaaggaa acttggaaagt
 601 ggtgggagtt ttcttggcca ttcacatgg cctactctgt tttactgtc ttcacaccc
 661 caaagcagca catttcaata acaaacacaa ggtttsacca ctgttcaata ccacccctc
 721 tttttgtaa acctgttagaa aagaggatcc taattttttgg tagmatccaa mtttacagcc
 781 aggataatata gagatggaaag aagggtcttg ggggaaagtc tccatgtggc cccgttaactc
 841 cataaaagctt accctgtttt cttttgtgt cttacttagg tttctccca tggaatgctt
 901 tccctggcaa ggtttgtggc tccaaacctt tttccatctg caaaacagct gaggtgagtg
 961 ggttattttgg gttattttac aaggagtag ctaataccat acaaattaca cccatggcc
 1021 tcaattttaa ggactgaaag tttccctttt ctggatttt aattagccga ttgccttctt
 1081 caacatgttg gctaaatgtt cctgagccaa tgagcataga agttaaaaaca cctttttctt

E. 1 aatttagcaca cagaaaggat atccaaacaca tacaagctg tnntcatgga ctacactgg
 61 gcatattact gctgttgcaaa gaaacatcc ttcttcctt tttcattttctc ctgcagttcc
 121 aaatgacctt ccacccctgtt attgctgcattt ttgtggggcc tgcagctaca ctggtttccc
 181 tggtgagtttgc actttgaatg atttggccaa gtaaatagggc ctgagatagt tttgggtaca
 241 gctattctgtt aaggcaagaa ggttagactgc tccatcctt gaaatgtgg aggaa

FIGS. 17C-E

1 aattctatac actatcacta tggctccact ttggatactc tccagtggat tttagttact
61 atatgaaat acctgggagg acctccctaac attattagaa ttgttatgat tataatacaa
121 ygcatagtcc caggcttgc tgatagtgtc acagtgcctt gtgaatgtag tgtgtcatt
181 gtgcagatta aaaacctaag gcactgaagg gtgaagtgtat ttatctgaag ttatTTATA
241 aagcaatgtat cagacaasct gagctcacag aactccctgg cccctactgc tgaggttcc
301 atacagagtc aagtaattc tcacctgtt aaacgaattt attcattaac caggggagag
361 ctctactgca tgatgtggct gtgtgtctac agcaagcacc ctatgactct aagtcactcg
421 gacatattga tggcggaaag cccaaatatt gttcaatttcc ctgaggaaaa ctcagtgcta
481 gatcaaacag aggtgtggaa taaatcttta tgatttgatt ctctgggct gggccatgag
541 acccatgtat cctcagagac atcggacttc cagtcagtg tatatggaga aagccaagcc
601 tggatgtac tgcttttgc agagcatggg tttttccctt atttagttt gattttttt
661 ctacccttcc tcattcccaa agggattga ggaggagtg ctttcttcc tacttcatt
721 cacatctctt ctctgttcc ctacagtc ccttcatgt tgctgcaact tacaacttgg
781 ccgtccttaa actcatgggc cgaggccca agttctgtc ccccgtagaa atccccctt
841 ctctaatagc gaggtctaa ccacacagcc tacaatgtc cgtctcccat ctaatctt
901 tgccttgcc accaactggc ccttcttta cttgtatgagt gtaacaagaa aggagagtct
961 tgcagtgatt aagggtctctt ttggactct ccccttttat gtacctttt tagtctttt
1021 gttcatagc tggttctgc tagaaatggg aaatgcctaa taatatgact tcccaactgc
1081 aagtccaaa ggaatgggg ctctaattga atttcaagc atctcctgag gatcagaaag
1141 taatttcttc tcaaagggtt cttccactga tgaaaacaaa gtggaaaggaa agatgtcag
1201 gtacagagaa ggaatgtctt tggtcttcc gccatctata ggggccaat atattcttt
1261 tgggtacaa aatggaaattt atctctgtc tetctattac actgaagata gaagaaaaaaa
1321 gaatgtcaga aaaacaataa gaggcttgc cccaaatctgc ctattgcagc tgggagaagg
1381 gggtaaaagc aaggatctt caccacaga aagagagcac tgaccccgat ggcgatggac
1441 tactgaagcc ctaactcagc caaccttact tacagcataa gggagctgat aatctgtgt
1501 gacgaagggg gcacatctggcc ttacacctcg ttagggaga gaaacagggt ctgtcagca
1561 tcttctact cccttctct tgataacagc taccatgaca accctgtgtt ttccaaggag
1621 ctgagaatag aaggaaacta gcttacatga gaacagactg gcctgaggag cagcgttgc
1681 tgggtctaa tgggttaacc tgagatggcc ctctgttgc cacaggatag ataactctt
1741 ggatgcatg tttttttt tggatattag tgggttactc tggcctctgt catacttca
1801 caatgggtc catttcatgg ggtattatcc attcagtc catgttgc tgggttactc
1861 tgatttggtt tagaatgtat cacatttcat gtattccat ttgttattt cttatTTGGG
1921 gttgcatcag aaatgtctgg agaataattt tttgattatg actgtttttt aaacttagaa
1981 aattggacat taagcatcac aaatgtatatt aaaaattggc tagttgaatc tattggatt
2041 ttctacaagt attctgcctt tgcagaaaca gattttggta atttgaatct caatttgagt
2101 aatctgatcg ttctttctag ctaatggaaa atgattttac ttagcaatgt tatcttgggt
2161 tgtaagagt taggtttac ataaaggta ttttctccctg atatagatca cataacagaa
2221 tgcaccagtc atcagctatt cagttggta gcttccagtc atcagctatt cagttggtaa
2281 gcttcccagg aaaaaggaca ggcagaaaga gtttggagacc tgaatagtc ccagatttca
2341 gtcttttaat gttttgtt actttgggtt aaaaaaaaaaa aaagtctgtat tggttttaat
2401 tgaaggaaag atttgcatac cagttttttt gttgtttaaga gttgtgtgt tctttttccc
2461 caaagtgggt tcagcaatatt ttaaggagat gtaagagctt taaaaaaga cacttgatcat
2521 ttgtttcaa accagtatac aagataagct tccaggctgc atagaaggag gagaggaaaa
2581 atgtttgttta agaaaccaat caagataaag gacagtgaag taatccgtac ttgtgtttt
2641 gttttgattt aataacataa caaataacca acccttccctt gaaaacctca catgcataca
2701 tacacatata tacacacaca aagagagttt atcaactgaa agtgttccctt catttctgtat
2761 atagaattgc aattttaaaca caacataaagg ataaactttt agaaacttata tttacaaagt
2821 gtatTTATA aaattaaaga aaataaaaatt aagaatgttc tcaatcaaac atcggtcct
2881 ttgagtgaat tggttctattt gacttcacaa tagaaactta ataatcgatc ctttctcaaga

FIG. 17F

1 atggaaatgt tctgtatttg tgggtctga tgagataacc actaactgta gtgctattga
 61 qcatttggaaa catggctagt gtaatcaatg aacccaaattt ttaattttat ttaattgtaa
 121 ttaattttaa gtggccacat gcagggagtg actgctgcac tggacagcac ggctctaaat
 181 tgagcctttt ttcccttattt ggtgaggcat acttgcctt aagttgggaa gtctatttt
 241 ggaacctgct accaatgctg gtctcacact tgcaattctc agctgagcca agaggtaga
 301 gaaaggcat tttccattcc aagatctcac ttccttctgt gacactgagg aaactggcaa
 361 gtgatgtgaa ggctggagag cgtgtctgt atgctggctc tggcccttgc gctgtgttg
 421 actgacatag tttagtgcgt cccttgcgg ttccttcc tccacacttgc cctctcttag
 481 cacacctgac attcatctca tgacttccct aaaaacattt tttgggaaaca agaaactaac
 541 aaatcccaag tgaccttatca catataaaaa catacaggc agagtttggaa ttgcggtag
 601 aagaaaggaa ggttagacat taagaagaat ggtctggta tgacagtgt gagataatag
 661 aaacaggaaa aagaaatcta agttttttt cttttttaa gaaccaataa taattttct
 721 ctggacta gtcagtaggg ctgggttggg ttggaggaag cttacatattt ccatgaacaa
 781 gccttccct aaggctctgt aagtgtatcc gcccactga ttagccccata gaagaccctt
 841 caaagggtgg attcggaggg gggagtggg gggaaaggc ctgtaccagg cagctctgc
 901 tccattgctc tgggggggtg gggaaagacaa accctggta tcccctcaat ctgtagccct
 961 tttgtgtgag tgccctggcaa ggggtgacgtt gggctgttc tgcgggcaca gctcagcaa
 1021 ttaccggagt ggaggcaggc cccaggcagc actgcctcc aagatcttcc ttgggctt
 1081 tcagcagtaa ggggacatgc accccaaggc cctccacttgc gcctgacctt gtcgggggg
 1141 ctctctgtcc ccaggaaacag tagagatggc aagcttacatcg agacccttc tggccagctg
 1201 cctctgtcc ttcccttcc tcccttccctt ccaagtgtct tccagctatg caggtaaagac
 1261 atgtttttt tccgtccctg gggagaccc gaaaacagaa aggctagttt cctgggggtt
 1321 agtccttca aacatctca agttggata ttatcttctt aaaaacataga cctactgaca
 1381 tgcctccctt ctcagaaac cttccgtggg tgggtcttac agccttcaag atggagtcca
 1441 gactctttt tttttttggg acagagtttc cctctgttgc tcaggctggta gtgcgtggc
 1501 atgatctcggt ctcaactgcaa cctcagctc cctgggttcaa gcgatttcttca tgaattggcc
 1561 tcccaagtag cggagactac aggcgcctgc caccacaccc agctaaatttt gttttttt
 1621 ttctttttt tttttttgg gatttttagga cagacgggtt ttcacatgtt ggccaggatg
 1681 gtctcgatct ctggacctgc tgatccggcc gcctcagttt cccaaatgtac tgggattatg
 1741 ggcgtgagcc actgcacttag gcctaattttt tttatttta ttagagatgg ggtttcacca
 1801 tggggccag gtcggctgg aaccctgtac ctcaagtgtt ctggccctt cagcccttcca
 1861 aagttctgag attacaggca tgagccattt cgtctgaccc agactccttta atgtgactaa
 1921 ctccaggctt tccttggact acttcttact tgccttcca gctttgttctt ttccctctc
 1981 caatttggat aaaaataataa caaccttgc gagttctcat caggattaca taaaatgaa tttttttt
 2041 tatgttaacat gcttagcgt gcctgtccat agtaaatctc aataaaatgtt tttttttt
 2101 taatatcttgc tcatgtttga gactttgttgc tgcatatca ggcaccatgtt ggtttttt
 2161 aaggaaccccg tctgtcacgt gcagaggaga aaaaaacaga aagtttccca tcctcaggga
 2221 gccacccgtac tgacagggc acagtgcac tcaacttccat gttttttttt gaaaggcagcc
 2281 ttatcttca ttagctcaga atctgtacttgc agaaacacat ccacatagaa aaaaacaagg
 2341 aacttttccg ggtcagggtc cggggccac agtgggtgg aagatacagg ggaaggaaaga
 2401 gggaaataga gccatccccca ggggtggaaaga tctcagaaga gatttggga aacaaggat
 2461 gaacaaggac tgaatagtga gaagtgtatgg agagacagct aagtagatg gatgtcaaa
 2521 accaaaaaccc ttaagggttag aataggcagc aatttggcca agtcctaaaca gggaggcccc
 2581 taggaggatt caacctcaag atgctgtgcc acatttcaag agggaaaccta aaggctgggc
 2641 tgaagagtca gagatggctca cagctggcaa aqagatgggc agatgttgc agggatgtat
 2701 tgctaaaatg ttctgtccat gacattcaca gtatctctat aaccagatgc tttttgtcg
 2761 ttgttgttctt caagaaggaa acttggggcc ggggtgtggg gttttatggcc ataatccct
 2821 cgctttgggg ccaaggcagg cggatccatc gaggtcaggaa gttcggatggcc agcctggcc
 2881 acagtgtgaa acctcatctt tactaaaaat aaaaaatata gctggatgtcg gggtaggtg
 2941 cctgttaatgc cagctactcg ggaggctgag gcaggagaat cacttgaacc tgggaggcgg
 3001 agtttgcagg gaggcggagg ttgcgttgc ccaagattgc accactgcac tccagcctgg

FIG. 18

3061 gcgacagaga gtaagactgt ctcaaaaaat aaatgaataa ataaaaaggaa agaagaagaa
 3121 gaagaacaat tgcaatecctc cctggctcta gaatgtcatt taaaagtgcg gtgtcttctt
 3181 cttccctgt tttgaagcag cccttcctcat gacaggcttgc cttgcgaagg ttcccctctga
 3241 ccttaaatct cttcccttttgc gtgtcttgcg cagggcagtt cagagtgata ggaccaaagac
 3301 acccttatccg ggctctggtc ggggatgaag tggaaattgcc atgtcgata tctcctggga
 3361 agaacgctac aggcatggag gtgggggtgtt accggcccccc cttctcttagg gtgggttcata
 3421 tctacagaaa tggcaaggac caagatggag accaggcacc tgaatatcg ggccggacag
 3481 agctgctgaa agatgttatt ggtgaggaa aggtgactct caggatccgg aatgttaagg
 3541 tctcagatga aggaggtttc acctgttct tccgagatca ttcttaccaa gaggaggcag
 3601 caatggatt gaaatgatgaa ggtgagtagt gccatataat attaggatt aactgttggg
 3661 tggccaagaa caattattct ctcacttgat atgagatccc tcaacccaaa catctcagtc
 3721 ctgggaatga tttccataaa aatgtacaca tcaataaaaca gaaactcatg cttagggatg
 3781 tctgttgcatt cattattcag agtagcaagg aaattggat caaaatcaat qcctttgagt
 3841 aggttaagtgc cagaatgaac aatgttagcc atactgtgaa tattatgcag ggattaaaaaa
 3901 gattatttttgcacttagggcc agatgggttgggggtctt ctaaggattt attgagtgat
 3961 aagagcaagc tgctgttaga tacaaaaaaca aaaacaaaaac cctagggcat ggtgggttgc
 4021 ctcgcagcta ctcaggagcc tgagacggga ggctggcttgc agcccagggg tttgcagtt
 4081 cagttaggcata tgattgcacc actgcacttcc aacccgggttgc acagagcaaa gaccccttcc
 4141 cccactccctt acccgctctt aaaaaaaaaaaa aaaaaaaaaaaa aaaaaaaaaacc cttggccca
 4201 gcgcgcgtggc tcacccctgtt aatcccagca ctgtgggaggcccgagggtggg cagatcacaa
 4261 ggtcaggaga tcgagaccat cctggctaaa acggtaaaac cccgtctcta ctaaaaatac
 4321 aaaaaaaaaaaa aaaaaattta gccaggcatg ttagcaggcg cctgttagtcc cagctactcg
 4381 ggaggctgag gcaggagaat ggcgtgaacc cggaaagcggg gttgcagtg agccaaaatc
 4441 ctccactgc actccagcat gggggacaca gcgagactcc gtctaaaaaa aaaaaaaaaaaa
 4501 accctgttatt ttttgcac
 4561 gtgataaagc aagttaatca aatgtctaaa tataattata gaaaggagat gtcacccctt
 4621 ggctgtacctt ccacttatttc attctgcaga attgcagaat ttctttttt ttcccttct
 4681 ttctttttttttt acacagatgc tgcgtctgtt acccaggctg gagtgcata
 4741 gcgcgcgtggc cctccctgggt tcaagtgtt ctcctgcctc agcctccggat gtagctggga
 4801 ttacagggtgc ccaccaccac acccagcttac tttttgttattttttagatgag acagggttcc
 4861 acccagggtgttgc caagggttgc tctaaactcc tgacccctcagg tgatccactc gcctcagact
 4921 cccaaatgtgc tgggattaca ggcgtgatc atgggtggccgc gcctcagaat ttcatttca
 4981 acatgttttgc catgtatgggtt gattttggag aatattttt gctctatcgc aggatgatta
 5041 agatgtggac aaggtaagc cgatggaggg ggagcttgc aagttacttgc ctatttaatt
 5101 gaggaactaa actgttttgc gagecctgggg gtcagatcctt ctgccttttccctccctt
 5161 cctgcagtc aaacatcaga caattgtatca ctatgtatc ttggagggtgg gagtgaccat
 5221 tgcgtgtgc ggaccagaag atggcattgt atgttgcaca acaaagcact atttcttagag
 5281 actgcctgc aggatgttgc aatagcttta tttgttgc ttttttttttgc atacagctgt
 5341 ttttatttttttgc gaaatttttgc ttttgcggaaaa gtttgcatttttgc gtttgcatttttgc
 5401 gatcttttgc cttccctgc ttt
 5461 ccctcaggatcc caccac
 5521 atcccaaaaac ttt
 5581 agcctgcacca acatggagaa acccctgttgc tactaaaaac aaaaaatttttttttttttt
 5641 tggcgatcc ctgtatccctc agctggggagg ctgaggccagg agaattgcgtt gaccccccgg
 5701 ggcggaggttgc gcaatgttgc aagatcgttgc cattacactc cagtttttttttttttt
 5761 gaaactccatc tccaaaaaaa aaaaaaaaaaaa aacaaggaaag acaaaaaagaa aagcagctaa
 5821 agacttttgc tcaaggggaga aatgttgc ttttttttttttttttttttttttttttttttt
 5881 ttcccaacccctc ttctgttgc ttttttttttttttttttttttttttttttttttttttt
 5941 gtcttaggttgc tggagccagg aatgttgc aatatttttttttttttttttttttttttt
 6001 tatttgcacttgc ttt
 6061 ttt

FIG. 18 (cont.)

6121 tctttaaatt cttcattatg aaacataaaa acaaattgcca ggccggcag ctacgcctg
6181 taatcccagc actttggag gccgaagcgg gcagatcacc cgggtcagga gttcgagacc
6241 agcctgatca acatggagaa accccgttc tactaaaaaa tacaaaatta gctaggcggt
6301 gtggcacatg ccagtaatcc cagctacttg agagactgag gcaggagaat cgcttgaacc
6361 gggagggcaga ggttgcggg agccaagatc gcgcattgc actccagct gggcaacaag
6421 agaaaaactc tgtctaaaaa aaaaaaaaaacc acatacaaac cagagataat attataatga
6481 gcctccaagt gcctaccacc ttgtgcagc acttgtcaat ccagggacca cccacctcac
6541 cggctccccca ctcattacca ccctccccca ctcattact gaggtaaatc ctaggcagca
6601 ttagtatttttct ttttatttat tttgagacag gatctgttc tgtagccccag
6661 gctggagtgt agtggcatat ctctgctcac tgccgcctc gcctccggg cagaagccat
6721 cttccccaccc cagcctacat agtagctggg accacaggca cacacacca cactactgt
6781 atgtttgttta tttttgttag agactgggtt ttacatgtt gatcaggctg gtctcaaat
6841 ccttaggtca agcaatctc ccacctcgcc ctcccaaagt gctagaatta caggcgcag
6901 ccactgcacc cagcgaagaa cacttttaa aaaataataa ggcgggccc ggtggctcac
6961 acctgtatc ccagttactt gggagccaa ggagggcgaatc tcatgaggtc aagagattga
7021 gaccatctta agtaacatgg tggaaacccca tttctactac aaataaaaaa aaaaaattag
7081 cctggcgtgg tggcaggcgc ctgtagttcc agctacttgg gagctgaggc aggagaatgg
7141 agtgaaccccg ggaggcggag ctgcagtgaa gctgagatca tgccactgca ctccccctcg
7201 gggcaacaga gtgagactcc caaaaaaaaaa aaaaaagcc cccctcccc acacacaata
7261 atataaataaa ataaataacc acaataactat tatcacatct taaaactca aaaaaattt
7321 cttatatatca tcaaataccccc agtttgggtt caaattttcc tgattgttc ataaatatac
7381 tcttacagtt ggtttctttt agcgagatc aatgagacc cactgttga ctttgcct
7441 taggggttcc cagggctgtt attttgttga cgacattttcc atttgtctat gtaatacgg
7501 cttccatgcc ctgtttttt ctgttaactc atagatgtgg aggtgcata gatcttgc
7561 ttgatattact ttggcaaata tagttcatca gtgatactct atacttctt tgctttaca
7621 tccggaggct gataatgtct gctttctct ttttctaatttttgcattt tttttttttt
7681 tgggggggttgg gggaaaaaaa acccttaagt acatactcgc taaaatcacat
7741 aacttccatt aagaacttga aagtaaagggt agctgcattt tcccttaggg aacacaatga
7801 tagacaggag ccttagtcta cagcttgaag gattgttaattt atacctaagc aaccctctg
7861 gaccagttt atgttatttag ctgtgtatgtt tccctacattt tgatgtcatt
7921 agctccctta aagcagagat caagatggaa agggcttcag ctgcagcatg
7981 attagagtgg ggcttttgg tgcgtgaggag cagacctaga atggaaata gatggagcc
8041 acagaagtga aggtccccctt ccccttatttgc tcaacctact ccacatctcc
8101 atctgttcaag ttactgtatc ctgtgtaaagc tacctttttt ttctttttttt
8161 ttatattttt ttttttttttgcattt ttttttttttgcattt
8221 tgcattctcg gtcacttgc cccttccaaact cccaggttca tgcatttctc
8281 cttccaaatgtt gctggatttca caggctgcac caccatgtct ggtaattttt
8341 gtagagagag gtttccacca ttttttttttgcattt
8401 ccacccaccc tggctccccca aatgttggg attacagggt
8461 aaactaccc ttttttttttgcattt
8521 cttccgcaag ctgtatgttgc tgcatttgc
8581 agtgtctcac ttttttttttgcattt
8641 tccacccctccaggcttgc
8701 gtttgcattt
8761 ttttttttttgcattt
8821 cccaaatgtt gtttgcattt
8881 acaccaatgtt
8941 aggtgttgc
9001 gtttgcattt
9061 ttttttttttgcattt
9121 ctcacccctccaggcttgc

FIG. 18 (cont.)

FIG. 18 (cont.)

12241 actgtatcac ctgtacttat atttctgctt tacaaactca gnatgtttcc atgagtacag
 12301 aacatgacta atcagagaag acctcataga ggaatagaaa agccaccaag ccccactagg
 12361 aattgacccc tcaaggacat gtttcttagc ctttttgc actgcagatt gcccaatgcc
 12421 taaagataat ggcaacagaa gaccaaccaa atatttgta gataaatgtt gcagacacta
 12481 gaaggtgtca tttagggcaca gatggtacct tetctgagca aacttcctc acagctcc
 12541 cttcccgaggc tggatgtac tctactcttgc tcacccggca cacagagttc tatctgacga
 12601 ttttagaaat tagaccatg tggggaccac acacacacac atctttacac acccaaagag
 12661 gaggaaatgt atctttgtt tggaggactt gactatgaaa ggtttaact ctttttgta
 12721 ccatgaatct ctctggcaact ccagtgaatg ctaaaggacc ctttgcaga atgttttaa
 12781 atatacacat aaaatagaac acataggatt gaaaaacaa tcattgtact aaaatacagt
 12841 tatcaacccga taatcacatt tggatatacg taacataat gtttctttt ttttttttg
 12901 gaggcagagt ttggcttttgc tcacccggc tggagtgcaaa tgccgcgatc taggctact
 12961 gaaacctctg cttccgggt tcaagcgatt ctcagccctc tggatgtctg ggattacagg
 13021 tggccggccac cacacccagc taattttgtt attttagta gagacttaggt ttcacccagg
 13081 tggccaggt ggcctcgaac tcctgcaccc aggtgatcca cttgccttgg cttcccaaag
 13141 tgcgtggatt acgggcatga gccaccgtgc cggccatata atattttttt agccaaagta
 13201 atacattaag taatgttagca gcaagtctaa taacctgttaa ttcttttctt tttttttt
 13261 tttttttttt ttttagatga agttttttt agatggatg caatggcaca atctggctc
 13321 actgcaacccctt ccaccccttgc ggttcaagcg attctctgc ctcagccctc caagttgt
 13381 gaactacagg cgcatggccac catggccagc taattttgtt attttagta gagacgggt
 13441 ttccacatgt tggccaggct ggttgcac ccctgcaccc aggtgatctg cttgccttgg
 13501 cttccaaag tgcgtggatt acaggcatga gccaccaggc. ccagcccaat aaccttaat
 13561 ttcaacatataaatac aaacagtatt tcaagatttc tgcataact ctaatggaa
 13621 tgaazacatc tggcttcc attggtaatt aagtccacagg tactgctcat attgtggta
 13681 gttgtaaaat gtttggttt gtttggttt tccaaagact tgggggaatg ggtgtggtg
 13741 ggatcaacaa gagtcttgc tggccggccca ggctggatg cagggggagg atctggctc
 13801 actgcaacccctt ccgcctccca ggttcaagcg attctctgc ctcagccctc tgatgt
 13861 gcattacagg catgtggccac cacggccagc taatttttac attttagta gagatgggt
 13921 ttccacatgt tggcttggct ggttgcac ccctgcaccc agatccacc cgtctggac
 13981 tcccaagatg ttgggattac aggcatgac caccacactt ggcagttttt acatttttaa
 14041 tggaaagaaa tggtaatcc agtttttggaa aataaggagg cagttttt ctcattcaag
 14101 ttcatgaatct tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 14161 ggaacccaaa aagacagggt ggtggggcat gggggggac acatgttaat ccctgtttt
 14221 tctggtaac aattcagatc cccacttttctt ggggtggcc tgcgtggaa taaccctgtt
 14281 tggtaatgttcccggttgc gaccccttgc tgccttgc tttttttttt tttttttttt
 14341 tcgaagacta gcaagggtcag tggctggca gcaaggcaaga ccaccaataa gtggggacc
 14401 aagtcaagtc tgaatggaa gccaaaagag aatagaacca ggactcaaga ttagggggc
 14461 tgggatttcc ttatccctt gttttttttt tttttttttt tttttttttt tttttttttt
 14521 agagaaccac ttactggatc tggggatcc cccacttgc gggggatgg tttttttttt
 14581 aaatgtccat aggaggatg tggggatgg tttttttttt tttttttttt tttttttttt
 14641 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 14701 taagcagaga ataaaaagcc agggaaaggga gacagaagca acaagaggaa gaggccggc
 14761 attggggat cacattccca ggg
 14821 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 14881 agaagctgga ggcacttcta tttttttttt tttttttttt tttttttttt tttttttttt
 14941 ggaatccaaac cttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 15001 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 15061 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 15121 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 15181 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt
 15241 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt

FIG. 18 (cont.)

15301 gatttccctt cttctgtcat ctaccccttc tcttcatttt cccatTTTA ttacccttct
15361 ttccatTTCT ctctccagTC trccacCTgg aagccCTC tgGctaaggA caggcaggTG
15421 cccCTCTC catcagAGGA caccTGTact ggAGAGCAAC acaggATGGT ctctGCCATG
15481 aactggaggc caggaatCTC ctcaCTgAAA attacAGTat ggtAACTTTG caaatGGTGG
15541 ttgtttCTTC caagactCCA gccCTGATTG cgcaaaACTG aaaggCATGT gaaggGAAGG
15601 aagagGAAGA gtGCAAACa ttaAGAGAG agCTGAGTGA GCTGAAGAGT gaggatATGA
15661 gtagccccAA cccAAACCTG gagatGGGGa gaaACCTACA gaataCTAGC cagAGCTCT
15721 ctttGTTCTG gcAGCCTACT aggacCTGG ggaAGCAAAA acgAAAGCTG ggcaACATGC
15781 ctgtCTTAGA atGTTTCTCT tcaTCTTACA catTTCCAC aggtCTCAGA atCTTTCTT
15841 cctCTCATCC ttttCTCTA tCTACATATC tATCAGAGTA TCCACTGTTT atTCACAAAC
15901 tactACTTGa tggTCAGACA caAACAAACa agCTAGTGC taATTAATAA agatacGAGT
15961 tttggccGGG tgcggTGGCT cacGCTGTa atcccAGCAC tttggGAGGc CGAGGCGGGC
16021 gaatcacGAG gtcAGGAGT caAGACCAGC ctggccaaca tggTAAACC ccATCTCTA
16081 taaaaataca aacaATTAAC tgAGCATAGT ggtgggcacc tataatacca gctactCCGG
16141 aggCTGAGGC aggAGAAATCG CTGAAACCCa ggaggcAGAG gttGAGTGA gctgAGATCG
16201 cGCCACTGCA ctCTAGCCGG agtgACAGAG taAGACTCTG tCTCAAAAT aaATAATAA
16261 ataaATAAAT aaATAAATAA ataaATAAAA aATAATAATA caAGTTTCA taAGCACACT
16321 tctaACCCCT tGTTTTfAT gtATTTCTT ctttatCCAC gcACCTGTCT ccCTCTACTC
16381 cagCCTCATT ACCCCAGAGG tcAGTCTCA gggAAACTAA acacaAAAGAA agaGCTAGT
16441 cagaAAAGGCC ATTtTTTAT gttcaAGAT gctcaCTGCC tCTTTGTTT tGTCTCTT
16501 gcaggCCCTC tCTCTTAGGC ctCTTCTCCT gggggATGG atCTTGGGG GAGATRgATC
16561 acCTTCCATGC ttCCATTCCT ccccAGCCAT agtGGGACA tcatGAGAGA AGCCAAAGCC
16621 ctggcccAGG atCACCCGGC ATTtATGGTg gctGCTCTGG cacAGGTCTC tgcCTTATA
16681 gcccCTCCAG tgATCCATAA gggCCCTTTT cttccccAAAG gagAGGTcAC agatAGGGCA
16741 aaggtagCTC ttCTGTTCC agTGGGTCTG ctggTGTCTG accAGCTGG AAAATGAGET
16801 gaaAGACTTG ctGCAATGGA agCAGTAGT gggcgCTCT gtGAGGTGG CTTCTGTG
16861 tctggagaga taggATTTCT tGCTAAAGT cAAAGAACAA tggggGCAAC agaAGACATT
16921 gagTCTTgAg ggCTTCACTG gatGAGAGT ggatCTGGCA tCTGACAGA gggttCCAGT
16981 gatgggtGCC tgggtCTGG tCACAGGTGc ttggTTCTTA agtACAGATG cctggTTCTG
17041 gcccataGGa ccCTCAgTTc taaATATGGG ttCCtGGGAC ctggCCACTG gtGcatGGT
17101 cacatCCAAA agCCCTGGA tggACCTCTG gcttCTGGCG atGGGTGTCT ggaattcAGC
17161 ctgggtGCCt ggaATTCtCA aagtACACTC ctggTTCTCA tCCACTGGCT cctggTTTG
17221 gtgtatCTTC tggTGGCgtT tgAGCTGAGA ctggTCCCGG aagCTCTTCC cacacacAGA
17281 gcatGAATGG gcccGTTAAC ccAGATGGAC gggGCGGTGA cGACTTAGTC cagaAGACATC
17341 acAGTAGGTC ttGTCAAGA gctGTCaaca gaAGGGCCTC tCCCCAAGAT gcatGCGTCT
17401 gtGATAGTGT AgggACTTGG ggCTCCGAAA caACTTCCCA cACTGACTGC agCTGTTAGT
17461 cagCTTGGGA ttGTGAACAA actGGTGGCT atAGAGGTAG gagcGCGCTGC tggAAACATT
17521 ggcacAGGTG tagAAAAA

FIG. 18 (cont.)

1 tttgtatgtc attgcaggat tcagtcttc cagttgtca tctatggAAC tgcctttc
61 ttcttcctt atggggccct cctgctggct gagggcttct acaccacccgg cgctgtcagg
121 cagatcttg gcgactacaa gaccacccatc tgccggcaagg geetgagcgc aacggtaaca
181 gggggccaga aggggaggggg ttacagaggc caacatcaag ctcatcttt ggagcgggtg
241 tgtcatgtt tggaaaatg gctaggacat cccgacaagg tgatcatcct caggatttg
301 tggcaataac aagggggtggg gggacaa

FIG. 14

1 ctgtatcagt gctccctcgac gcctcacttgt acttcacggja agagacttgg ttgactggcc
61 acttggagcg gaatcaggag acatccccaa ctcagagaga ctgagcccta gctcgcccac
121 ttgctggaca agatgatatt ctttaccacc ctgcctctgt tttggataat gatttcagct
181 tctcgagggg ggcaactgggg tgcctggatg ccctcgatcca tctcagccct cgagggcacg
241 tgtgtctcca tccccctgccg ttgcacttc cggatgagc tcagaccggc tgggtacat
301 ggcgtctggt atttcaacag tccctacccc aagaactacc cgccagtggt cttaagttcc
361 cgcacacaaag tggtccacga gagcttccag ggccttagcc gcctgttggg agacctgggc
421 ctacgaaaact gcacccctgct tctcagcacg ctgagccctg agctgggagg gaaatactat
481 ttccgaggtg acctgggccc ctacaaccag tacacccctt cggagcacag cgtcctggac
541 atcatcaaca cccccaacat cgtggtggcc ccagaatgg tggcaggaac ggaatggag
601 gtcagatgca tggtgccggc caactggccca gagctgcgcct ctgagctgag ctggctgggc
661 cacgaggggc tagggggac cactgttctg ggtcgctgc gggaggatga aggccacctgg
721 gtgcagggtt cactgttaca ttgcgtgcctt actagagagg ccaacggcca cggctgggc
781 tgtcaggctg ctttccccaa caccacccctt cagttcgagg ttacggccag tctgacgtc
841 aagtacccccc cgggtattgt ggagatgaat ttctctgtgg aggccattga gggctccac
901 gtcagctgc tctgtggggc tgacagcaac cgcgcaccgc tgctgactt gatgcgggat
961 gggatgggtg tgagggaggc agttgttgc agctgttacc tggatcttgg ggaggtgacc
1021 ccagcagagg acggcatcta tgcttgcctg gcaagaaatg cctatggccaa ggacaaccgc
1081 acgggtggggc tgagcgtcat gtatgcaccc ttggaaagccca cagtgaatgg gacgggtgg
1141 gcggttagagg gggagacagt ctccatctt tggatccacac agagcaaccc ggaccctatt
1201 ctcaccatct tcaaggagaa gcaatccctt ggcacgggtca tctatgagag tcagctgcag
1261 ctggaaactcc ctgcgtgtac gcccggggc gatggggagt actgggtgt agctgagaac
1321 cagttatggcc agagagccac cgccttcaac ctgtctgtgg agtttgcctt cataatcc
1381 ctggaaatgcg acgtgtgcgc ggcggagac accgtgcgtt gctgtgtgt ggtttttcc
1441 aacccggaaac cttcccggttgc ctttgcgttgc ctttcccgca acgtgactgtt gaaacggagaca
1501 gagagggagt ttgtgtactt agagcgcgc ggccttcttgc tcaccagcat cctcaacgc
1561 cgggggtcagg cccaaagcccc accccggcgtt atttgttaccc ccagggaccc ctacggcacc
1621 cagggcctcg agctgcctt ccaggagca caccggactga tggggccca aatcgccct
1681 gtgggtgctt tggtgccctt tgccatctt attggccattt tctgcttacat caccagaca
1741 agaagaaaaaa agaacgttac agagagcccc agtttcttccag cggggagacaa ccctcatgtc
1801 ctgtacagcc cccaaattccg aatctcttgg gcaaccttata agtatggagag tgagaagcgc
1861 ctgggggtccg agaggaggct gctgggcctt agggggggaaac ccccaagaact ggacccatc
1921 tattcccaact cagacccctgg gaaacggaccc accaaggaca gctacaccctt gacagaggag
1981 ctggctgagt acgcagaaat ccggatgttcaag tga

FIG. 10

1 masqkrpsqr hgskylatas tmdharhgfl prhrdtgild sigrffggdr gapkrgsgkd
61 shhpartahy gslpqkshgr tqdenpvhf fknivtprrt ppsqqkgrgl slsrfswgae
121 gqrpgfgygg rasdyksahk gfkgvdaqgt lskifklgr dsrsgspmar r

FIG. 24

1 mglleccarc lvgapfaslv atglcffgva lfcgcgheal tgtekliety fsknyqdyey
61 linvihafqy viygatasfff lygalllaeg fyttgavrqi fgdyktticg kglsvatvtgg
121 qkgrgsrqgh qahslervh clgkwlgpd kityaltvvw llvfacsavp vyiyfntwtt
181 cqsiapkskt sasigslcad armygvlpwn afpgkvagsn llsicktaef qmtfhlfiaa
241 fvgaaatlvs lltfmiaaty nfavlklmgr gatkf

FIG. 26

1 maslsrpsslp sclcsfllll llqvsssyag qfrvigprhp iralvgdeve lpcrispgkn
61 atgmevgwyr ppfsrvvhly rngkdqdgdq apeyrgrtel lkdaigegkv tlrirnvrfs
121 deggftcffr dhsyqeeam elkvedpfyw vspgvlvlla vlpvllqit lgivflclqy
181 rrlrgklaei enlhrtfdph flrvpcwkit lfvivpvlgp lvaliicynw lhrrlagqfl
241 eelrnpf

FIG. 23

Antigenic Specificity of Immunoprotective Therapeutic Vaccination for Glaucoma

Sharon Bakalash,¹ Anat Kessler,² Tal Mizrahi,¹ Robert Nussenblatt,³ and Michal Schwartz¹

PURPOSE. To investigate the antigenic specificity of the immune neuroprotective mechanism that can protect retinal ganglion cells (RGCs) against death caused by high intraocular pressure (IOP).

METHODS. A unilateral increase in IOP was induced in rats by argon laser photoocoagulation of the episcleral veins and limbal plexus. Rats with high IOP were immunized with glatiramer acetate (Cop-1, a synthetic copolymer) or with myelin-derived or uveitogenic peptides. When the steroid drug methylprednisolone was used, it was administered intraperitoneally every other day for 12 days.

RESULTS. Vaccination with myelin-derived peptides that reside in the axons failed to protect RGCs from death caused by high IOP. In contrast, IOP-induced RGC loss was reduced by vaccination with R16, a peptide derived from interphotoreceptor retinoid-binding protein, an immunodominant antigen residing in the eye. The benefit of protection against IOP-induced RGC loss outweighed the cost of the monophasic experimental autoimmune uveitis (EAU) that transiently developed in a susceptible rat strain. Treatment with methylprednisolone alleviated the disease symptoms, but caused further loss of RGCs. Cop-1 vaccination was effective in both EAU-resistant and EAU-susceptible strains.

CONCLUSIONS. To benefit damaged neurons, immune neuroprotection should be directed against immunodominant antigens that reside in the site of damage. In a rat model of high IOP, RGCs can benefit from vaccination with peptides derived from proteins that are immunodominant in the eye but not from myelin-associated proteins. This suggests that the site of primary degeneration in IOP-induced RGC loss is in the eye. Cop-1 vaccination apparently circumvents the site-specific barrier and provides protection without risk of inducing autoimmune disease. (*Invest Ophthalmol Vis Sci*. 2003;44:3374–3381) DOI: 10.1167/iovs.03-0080

Recent studies in our laboratory, using rat and mouse models of optic nerve injury and glutamate toxicity, have led our group to postulate that autoimmunity represents the body's mechanism of protection against harmful self-components,^{1–3} such as those produced in toxic amounts as a result

of injury to the central nervous system (CNS).⁴ An autoimmune disease might be the result of a failure to control properly the injury-induced autoimmune response, the purpose of which is essentially beneficial.^{5,6}

While investigating the possible role of the autoimmune response as the body's protective mechanism against destructive self-compounds induced by CNS injury, our group showed that the antigenic specificity of the relevant protective autoimmune T cells in rats or mice appears to vary with the site of the insult and the antigen(s) presented to the T cells at that site.^{7–9} Further studies suggested that the one of the functions of the evoked immune response is to help the resident immune cells (microglia) clear the injured site of cell debris and other deleterious matter, such as breakdown products of degenerating nerves^{10,11} (Shaked et al., unpublished data, 2003). In the course of studies seeking to boost the spontaneously evoked protective autoimmune response without risking an autoimmune disease, it was discovered that this goal could be achieved by vaccination with copolymer-1 (Cop-1; Copaxone; Teva Pharmaceutical Industries, Petah Tikva, Israel),¹² which appears to operate as an antigen that cross-reacts with a wide range of self-reacting T cells.^{10,13} Vaccination with Cop-1 effectively protects retinal ganglion cells (RGCs) against death initiated in the axons by mechanical injury or in the cell bodies by glutamate toxicity or high intraocular pressure (IOP).^{2,12} In contrast to the effect of Cop-1, the immune response evoked by myelin-associated antigens is protective only from death induced by axonal injury,^{14,15} and not from direct death of the RGCs caused, for example, by local injection of glutamate.²

Our recent study of IOP-induced RGC death in rats showed that strain differences in the ability to withstand the consequences of high IOP are immune-related.¹⁶ In T-cell-deficient rats of a strain in which naïve rats are relatively resistant to IOP-induced death, the constitutional resistance was restored by replenishment of the rats with splenocytes from a matched donor that had the appropriate ability to harness a T cell-mediated protective activity.¹⁶

Accumulating evidence in our laboratory suggests that the antigenic specificity of immune intervention by therapeutic vaccination for regrowth is dependent on the site of insult rather than the type of injury. It was therefore of interest to examine which of two types of self-antigens, those residing in the nerve or those residing in the eye, would be beneficial as a therapeutic vaccine for the treatment of RGCs under stress induced by high IOP. Our results showed that vaccination with myelin-derived peptides did not lead to protection in a rat model of IOP-induced RGC death. In contrast, R16, a peptide derived from interphotoreceptor-retinoid binding protein (IRBP), an immunodominant antigen residing in the eye, conferred significant protection against the RGC death associated with high IOP. Vaccination with R16, known to induce monophasic experimental autoimmune uveitis (EAU) in susceptible rats, caused some RGC damage in immunized EAU-susceptible rats with normal IOP but if these rats had been subjected to an increase in IOP, this cost was outweighed by the vaccination's protective benefit. Moreover, in rats that are not susceptible to EAU, no such cost was incurred.

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TABLE 1. Uveitis Scores

Flare in Anterior Chamber	Cells in Anterior Chamber	Iris Hyperemia Scale	Conjunctival Congestion Grading
0, Absent	0, 0-7	0, Normal iris, no hyperemia	0, Normal, without perlimbal injection
+1, Faint flare	+1, 7-10	+1, Minimal involvement of secondary vessels	+1, Palpebral and confined perlimbal congestion
+2, Moderate flare	1-2+, 10-15	+2, Minimal involvement of both secondary and tertiary vessels	+2, Palpebral with at least 75% of perlimbal region
+3, Marked flare (details of the iris and lens are hazy)	+2, 15-20	+3, Moderate involvement of secondary and tertiary vessels with slight swelling of iris stroma	+3, Dark red congestion with petechiae
+4, Intense flare (with large amounts of fibrin)	+3, 20-50 +4, +50	+4, Marked involvement of secondary and tertiary vessels with swelling of iris stroma accompanied by hyphema	+4, Confluent, dark petechiae of at least 50% of the conjunctival area

Hogan MH, Kimura SJ, Thygeson P. Signs and symptoms of uveitis. *Am J Ophthalmol*. 1959;47:15.

MATERIALS AND METHODS

Animals

Inbred adult male Lewis and Sprague-Dawley (SPD) rats (8 weeks old, average weight 300 g) were supplied by the Animal Breeding Center at The Weizmann Institute of Science. The rats were maintained in a light- and temperature-controlled room and were matched for age and weight before each experiment. All animals were handled according to the regulations formulated by the International Animal Care and Use Committee (IACUC) and according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of High IOP

Blockage of aqueous outflow causes an increase in IOP, which results in death of RGCs.^{2,16-18} An increase in IOP was achieved in the right eyes of deeply anesthetized rats (ketamine HCl 50 mg/kg, xylazine HCl 0.5 mg/kg, injected intramuscularly) by blocking the aqueous outflow in the eye with 80 to 120 applications of blue-green argon laser. The laser beam, which was directed at three of the four episcleral veins and at 270° of the limbal plexus, was applied with a power of 1 watt for 0.2 seconds, producing a spot size of 100 µm at the episcleral veins and 50 µm at the limbal plexus. At a second laser session 1 week later, the same parameters were used, except that this time the spot size was 100 µm for all applications, and the radiation was directed toward all four episcleral veins and 360° of the limbal plexus.²

Measurement of IOP

Most anesthetic agents cause a reduction in IOP,¹⁹ thus precluding reliable measurement. To obtain accurate pressure measurements while the rat was in a tranquil state, we injected the rat intraperitoneally (IP) with 10 mg/mL acepromazine and measured the pressure in both eyes 5 minutes later with a handheld tonometer (Tono-Pen XL; Automated Ophthalmics, Ellicott City, MD), after applying benoxinate HCl 0.4% to the cornea. Because of the reported effect of anesthetic drugs on IOP measured by the tonometer,¹⁹ we always measured at the same time after acepromazine injection and recorded the average of 10 measurements made in each eye.

Morphologic Assessment of Retinal Damage Caused by the Increase in IOP

The hydrophilic neurotracer dye dextran tetramethylrhodamine (Rhodamine Dextran; Molecular Probes, Eugene, OR) was applied directly into the intraorbital portion of the optic nerve. Only axons that survive the high IOP and remain functional, and whose cell bodies are still viable, can take up the dye and demonstrate labeled RGCs.⁴ The rats were killed 24 hours after dye application, and their retinas were excised, whole-mounted, and preserved in 4% paraformaldehyde. RGCs

were counted under magnification of ×800 in a fluorescence microscope (Carl Zeiss Meditech; Oberkochen, Germany) by an observer who was blinded to the identity of the retinas. Four fields from each retina were counted, all with the same diameter (0.076 mm²) and located at the same distance from the optic disc.^{3,20} Eyes from untreated rats were used as a control.

Antigen Purification

The following peptides were synthesized by the Synthesis Unit at the Weizmann Institute: R16 sequence 1177-1191 of bovine IRBP (ADGS-SWEGVGVPDV); a nonencephalitogenic altered peptide, amino acids 87-99, derived from an encephalitogenic peptide of myelin basic protein (MBP) by replacing the lysine residue 91 with glycine (A91; VHFFANIVTPRTP)^{21,22}; and a Nogo-derived peptide (AS472; SYD-SIKLEPENPPPYEEA), a myelin-associated growth inhibitory protein²³⁻²⁵ recently found to be protective when used as a vaccine in rat models of spinal cord injury and optic nerve crush.⁸

Active Immunization with the Peptides

Rats with a laser-induced increase in IOP were immunized, immediately after the first laser session, with R16 (30 µg), A91 (75 µg), AS472 (100 µg), or Cop-1 (100 µg) or were injected with phosphate-buffered saline (PBS) as a control. All compounds were emulsified in complete Freund's adjuvant (CFA) containing 2.5 mg/mL *Mycobacterium tuberculosis* (Difco, Detroit, MI).⁸ A total volume of 100 µL was injected subcutaneously into each rat at the root of the tail.¹²

Assessment of Clinical Signs of Uveitis

Both eyes were examined under a microscope. The cornea, flare, and cells in the anterior chamber and iris hyperemia were evaluated according to the scale presented in Table 1. The mean value for each parameter was calculated and graded. The examination was repeated every other day for 3 weeks.

Steroid Administration

Methylprednisolone (MP; Solu-Medrol, 125 mg/mL, Pharmacia & Upjohn, Puurs, Belgium) was injected IP at a dosage of 30 mg/kg.²⁶ Rats were injected on day 0 and again on days 3, 6, 9, and 12.²⁷

RESULTS

Failure of Myelin-Derived Peptides to Evoke Neuroprotection

T-cell-dependent survival of RGCs was manifested in all eye models tested. Moreover, the T-cell-dependent neuroprotective mechanism was found to be amenable to boosting, either

Antigenic Specificity of Immunoprotective Therapeutic Vaccination for Glaucoma

Sharon Bakalash,¹ Anat Kessler,² Tal Mizrahi,¹ Robert Nussenblatt,³ and Michal Schwartz¹

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Recent studies in our laboratory, using rat and mouse models of optic nerve injury and glutamate toxicity, have led our group to postulate that autoimmunity represents the body's mechanism of protection against harmful self-components,^{1–3} such as those produced in toxic amounts as a result

of injury to the central nervous system (CNS).⁴ An autoimmune disease might be the result of a failure to control properly the injury-induced autoimmune response, the purpose of which is essentially beneficial.^{5,6}

While investigating the possible role of the autoimmune response as the body's protective mechanism against destructive self-compounds induced by CNS injury, our group showed that the antigenic specificity of the relevant protective autoimmune T cells in rats or mice appears to vary with the site of the insult and the antigen(s) presented to the T cells at that site.^{7–9} Further studies suggested that the one of the functions of the evoked immune response is to help the resident immune cells (microglia) clear the injured site of cell debris and other deleterious matter, such as breakdown products of degenerating nerves^{10,11} (Shaked et al., unpublished data, 2003). In the course of studies seeking to boost the spontaneously evoked protective autoimmune response without risking an autoimmune disease, it was discovered that this goal could be achieved by vaccination with copolymer-1 (Cop-1; Copaxone; Teva Pharmaceutical Industries, Petah Tikva, Israel),¹² which appears to operate as an antigen that cross-reacts with a wide range of self-reacting T cells.^{10,13} Vaccination with Cop-1 effectively protects retinal ganglion cells (RGCs) against death initiated in the axons by mechanical injury or in the cell bodies by glutamate toxicity or high intraocular pressure (IOP).^{2,12} In contrast to the effect of Cop-1, the immune response evoked by myelin-associated antigens is protective only from death induced by axonal injury,^{14,15} and not from direct death of the RGCs caused, for example, by local injection of glutamate.²

Our recent study of IOP-induced RGC death in rats showed that strain differences in the ability to withstand the consequences of high IOP are immune-related.¹⁶ In T-cell-deficient rats of a strain in which naïve rats are relatively resistant to IOP-induced death, the constitutional resistance was restored by replenishment of the rats with splenocytes from a matched donor that had the appropriate ability to harness a T cell-mediated protective activity.¹⁶

Accumulating evidence in our laboratory suggests that the antigenic specificity of immune intervention by therapeutic vaccination for regrowth is dependent on the site of insult rather than the type of injury. It was therefore of interest to examine which of two types of self-antigens, those residing in the nerve or those residing in the eye, would be beneficial as a therapeutic vaccine for the treatment of RGCs under stress induced by high IOP. Our results showed that vaccination with myelin-derived peptides did not lead to protection in a rat model of IOP-induced RGC death. In contrast, R16, a peptide derived from interphotoreceptor-retinoid binding protein (IRBP), an immunodominant antigen residing in the eye, conferred significant protection against the RGC death associated with high IOP. Vaccination with R16, known to induce monophasic experimental autoimmune uveitis (EAU) in susceptible rats, caused some RGC damage in immunized EAU-susceptible rats with normal IOP but if these rats had been subjected to an increase in IOP, this cost was outweighed by the vaccination's protective benefit. Moreover, in rats that are not susceptible to EAU, no such cost was incurred.

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TABLE 1. Uveitis Scores

Flare in Anterior Chamber	Cells in Anterior Chamber	Iris Hyperemia Scale	Conjunctival Congestion Grading
0, Absent	0, 0-7	0, Normal iris, no hyperemia	0, Normal, without perilimbal injection
+1, Faint flare	+1, 7-10	+1, Minimal involvement of secondary vessels	+1, Palpebral and confined perilimbal congestion
+2, Moderate flare	1-2+, 10-15	+2, Minimal involvement of both secondary and tertiary vessels	+2, Palpebral with at least 75% of perilimbal region
+3, Marked flare (details of the iris and lens are hazy)	+2, 15-20	+3, Moderate involvement of secondary and tertiary vessels with slight swelling of iris stroma	+3, Dark red congestion with petechiae
+4, Intense flare (with large amounts of fibrin)	+3, 20-50 +4, +50	+4, Marked involvement of secondary and tertiary vessels with swelling of iris stroma accompanied by hyphema	+4, Confused, dark petechiae of at least 50% of the conjunctival area

Hogan MH, Kimura SJ, Thygeson P. Signs and symptoms of uveitis. *Am J Ophthalmol*. 1959;47:15.

MATERIALS AND METHODS

Animals

Inbred adult male Lewis and Sprague-Dawley (SPD) rats (8 weeks old, average weight 300 g) were supplied by the Animal Breeding Center at The Weizmann Institute of Science. The rats were maintained in a light- and temperature-controlled room and were matched for age and weight before each experiment. All animals were handled according to the regulations formulated by the International Animal Care and Use Committee (IACUC) and according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of High IOP

Blockage of aqueous outflow causes an increase in IOP, which results in death of RGCs.^{2,16-18} An increase in IOP was achieved in the right eyes of deeply anesthetized rats (ketamine HCl 50 mg/kg, xylazine HCl 0.5 mg/kg, injected intramuscularly) by blocking the aqueous outflow in the eye with 80 to 120 applications of blue-green argon laser. The laser beam, which was directed at three of the four episcleral veins and at 270° of the limbal plexus, was applied with a power of 1 watt for 0.2 seconds, producing a spot size of 100 μm at the episcleral veins and 50 μm at the limbal plexus. At a second laser session 1 week later, the same parameters were used, except that this time the spot size was 100 μm for all applications, and the radiation was directed toward all four episcleral veins and 360° of the limbal plexus.²

Measurement of IOP

Most anesthetic agents cause a reduction in IOP,¹⁹ thus precluding reliable measurement. To obtain accurate pressure measurements while the rat was in a tranquil state, we injected the rat intraperitoneally (IP) with 10 mg/mL acepromazine and measured the pressure in both eyes 5 minutes later with a handheld tonometer (Tono-Pen XL; Automated Ophthalmics, Ellicott City, MD), after applying benoxinate HCl 0.4% to the cornea. Because of the reported effect of anesthetic drugs on IOP measured by the tonometer,¹⁹ we always measured at the same time after acepromazine injection and recorded the average of 10 measurements made in each eye.

Morphologic Assessment of Retinal Damage Caused by the Increase in IOP

The hydrophilic neurotracer dye dextran tetramethylrhodamine (Rhodamine Dextran; Molecular Probes, Eugene, OR) was applied directly into the intraorbital portion of the optic nerve. Only axons that survive the high IOP and remain functional, and whose cell bodies are still viable, can take up the dye and demonstrate labeled RGCs.⁴ The rats were killed 24 hours after dye application, and their retinas were excised, whole-mounted, and preserved in 4% paraformaldehyde. RGCs

were counted under magnification of ×800 in a fluorescence microscope (Carl Zeiss Meditech; Oberkochen, Germany) by an observer who was blinded to the identity of the retinas. Four fields from each retina were counted, all with the same diameter (0.076 mm²) and located at the same distance from the optic disc.^{3,20} Eyes from untreated rats were used as a control.

Antigen Purification

The following peptides were synthesized by the Synthesis Unit at the Weizmann Institute: R16 sequence 1177-1191 of bovine IRBP (ADGS-SWEGVGVPDV); a nonencephalitogenic altered peptide, amino acids 87-99, derived from an encephalitogenic peptide of myelin basic protein (MBP) by replacing the lysine residue 91 with glycine (A91; VHFFANIVTPRTP)^{21,22}; and a Nogo-derived peptide (AS472; SYDSIKLEPENPPPYEEA), a myelin-associated growth inhibitory protein²³⁻²⁵ recently found to be protective when used as a vaccine in rat models of spinal cord injury and optic nerve crush.⁸

Active Immunization with the Peptides

Rats with a laser-induced increase in IOP were immunized, immediately after the first laser session, with R16 (30 μg), A91 (75 μg), AS472 (100 μg), or Cop-1 (100 μg) or were injected with phosphate-buffered saline (PBS) as a control. All compounds were emulsified in complete Freund's adjuvant (CFA) containing 2.5 mg/mL *Mycobacterium tuberculosis* (Difco, Detroit, MI).⁸ A total volume of 100 μL was injected subcutaneously into each rat at the root of the tail.¹²

Assessment of Clinical Signs of Uveitis

Both eyes were examined under a microscope. The cornea, flare, and cells in the anterior chamber and iris hyperemia were evaluated according to the scale presented in Table 1. The mean value for each parameter was calculated and graded. The examination was repeated every other day for 3 weeks.

Steroid Administration

Methylprednisolone (MP; Solu-Medrol, 125 mg/mL, Pharmacia & Upjohn, Puurs, Belgium) was injected IP at a dosage of 30 mg/kg.²⁶ Rats were injected on day 0 and again on days 3, 6, 9, and 12.²⁷

RESULTS

Failure of Myelin-Derived Peptides to Evoke Neuroprotection

T-cell-dependent survival of RGCs was manifested in all eye models tested. Moreover, the T-cell-dependent neuroprotective mechanism was found to be amenable to boosting, either

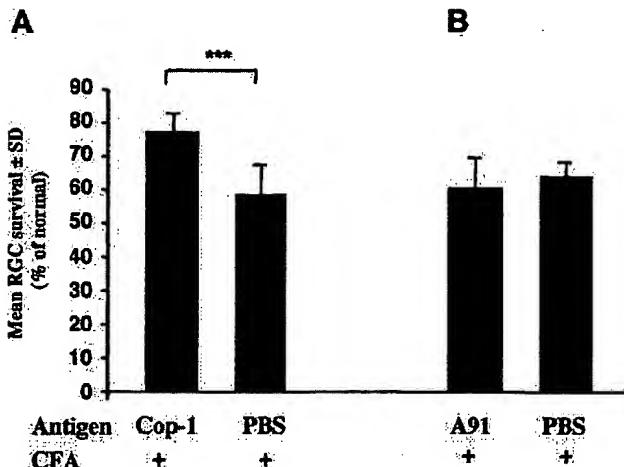


FIGURE 1. Immunization of Lewis rats with myelin-associated antigens, unlike Cop-1 immunization, failed to rescue RGCs from death induced by high IOP. Immediately after the first increase in IOP, adult Lewis rats were immunized with Cop-1 (A) or with A91 (B) emulsified in CFA (2.5 mg/mL). The same peptides, this time emulsified in incomplete Freund's adjuvant (IFA), were injected again 1 week later. Control rats were immunized with PBS in CFA and 1 week later with PBS in IFA. Whole-mounted retinas were excised 3 weeks after the first increase in IOP and 24 hours after injection of dye into the optic nerve. (A) The loss of RGCs in rats treated with Cop-1 was significantly smaller than in rats treated with PBS ($n = 7$ in each group; $***P < 0.0001$). (B) No significant difference was detected between the groups injected with A91 ($n = 7$) and with PBS ($n = 4$; $P = 0.3$). The data in (A) are from one of three and in (B) from one of two independent experiments with similar results. Note that two PBS control experiments are shown because the experiments were performed on different days, and thus required separate controls.

specifically by antigens residing in the lesion site, or nonspecifically by Cop-1, which seems to circumvent the tissue-specificity barrier. Thus, whereas myelin-derived peptides were effective in protecting against the degenerative consequences of an insult to optic nerve axons, they could not protect against a glutamate insult imposed directly on the RGCs. As a corollary, vaccination with uveitogenic peptides protected RGCs from damage induced directly by glutamate.

In the case of RGC loss caused by high IOP, it was not clear whether the primary site of degeneration is the optic nerve or the eye, and thus whether the antigenic specificity of the protective immune response would be directed to antigens residing in the nerve or the retina. To address this question, Lewis rats were subjected to a laser-induced increase in IOP and were vaccinated with A91 (a myelin-derived altered peptide that provides significant protection against RGC death after axonal injury) or the Nogo-derived peptide AS472⁸ on the day of the first laser treatment. Surviving RGCs were counted and expressed as a percentage of the number of RGCs in normal rats. As shown in Figure 1B, neither of these peptides could protect the RGCs against death caused by high IOP. In contrast, and as expected,² significant protection was obtained when these rats were vaccinated with Cop-1 as a positive control (Fig. 1A); the extent of RGC survival after vaccination with Cop-1 was $87.4\% \pm 4.9\%$ ($n = 7$) compared with $54.3\% \pm 8.2\%$ in PBS-injected control subjects ($n = 7$; $P < 0.0001$). Thus, Cop-1 vaccination reduced the extent of death caused by high IOP by more than threefold. These results suggest that the death imposed by high IOP in rats was, at least during the early stages, not in the nerve but in the RGCs, and thus that the observed degeneration of the optic nerve is a late event.

Immunization with Peptides Derived from Eye-Resident Proteins Protects against Retinal Ganglion Cell Death

To verify that the eye is the primary site of protection, we subjected Lewis rats to an increase in IOP and then immunized them with spinal cord homogenate or retinal homogenate. A beneficial effect of the vaccination was observed in the rats immunized with retinal homogenate but not in those immunized with spinal cord homogenate (Fig. 2). These results suggest that the specificity of the immune response in the case of an IOP-induced insult should be directed against antigens that are immunodominant in the eye. On the basis of our previous observation that immunodominant antigens in a particular damaged CNS tissue are capable of eliciting an autoimmune response that is protective in that tissue,⁹ we examined whether vaccination with an immunogenic peptide derived from the eye-abundant protein IRBP²⁸ would protect the RGCs from a direct IOP insult. The peptide used to test this hypothesis was R16, already shown by our group to provide protection in a rat model of glutamate toxicity in the eye.⁹ Immunization of Lewis rats with R16 resulted in significant protection of RGCs (Fig. 3A). Substantially more RGCs survived in the group vaccinated with R16 ($82.8\% \pm 7.8\%$, $n = 7$) than in the group injected with PBS ($58.9\% \pm 5.3\%$, $n = 6$; $P < 0.0001$). Thus, the IOP-induced loss of RGCs was reduced by threefold by vaccination with R16. Similar protection from RGC death induced by high IOP was achieved in SPD rats, a strain resistant to the induction of EAU, in which the extent of RGC survival was $83.4\% \pm 6.7\%$ ($n = 6$) after vaccination with R16, as opposed to $67.7\% \pm 5.4\%$ ($n = 4$) after injection of PBS ($P < 0.003$; Fig. 3B).

Effect of Steroids on RGCs

Because R16 is a peptide that can cause EAU in Lewis rats,^{29–31} it was important to determine whether the immunization itself could cause loss of RGCs in naïve rats with normal IOP. Vaccination of naïve Lewis rats with R16 emulsified in CFA caused EAU symptoms that were accompanied by 12% loss of RGCs compared with Lewis rats injected with PBS in CFA ($n = 6$; $P < 0.001$). A score for clinical symptoms of EAU was assigned to

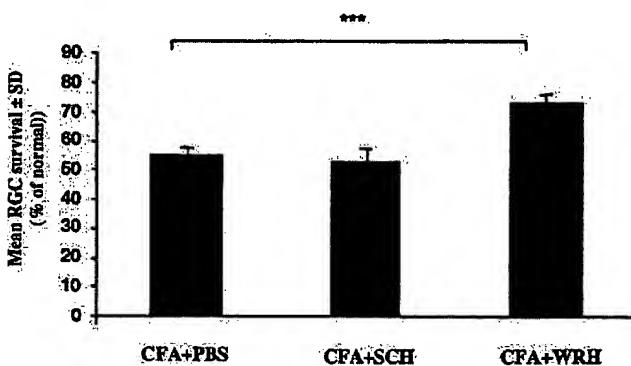


FIGURE 2. Protection of RGCs from IOP-induced death by immunization with retinal homogenate but not with spinal cord homogenate. Rats were subjected to two successive laser treatments to increase their IOP and immediately after the first treatment were immunized with spinal cord homogenate (SCH) or whole retinal homogenate (WRH) emulsified in CFA. Three weeks after immunization, the retinas were retrogradely labeled and then excised 24 hours later. Surviving RGCs were counted and expressed as a percentage of the number of RGCs in the nonimmunized control. Significantly more RGCs survived in the rats immunized with retinal homogenate than in control rats or rats immunized with spinal cord homogenate ($***P < 0.001$; $n = 5$ –7 rats per group).

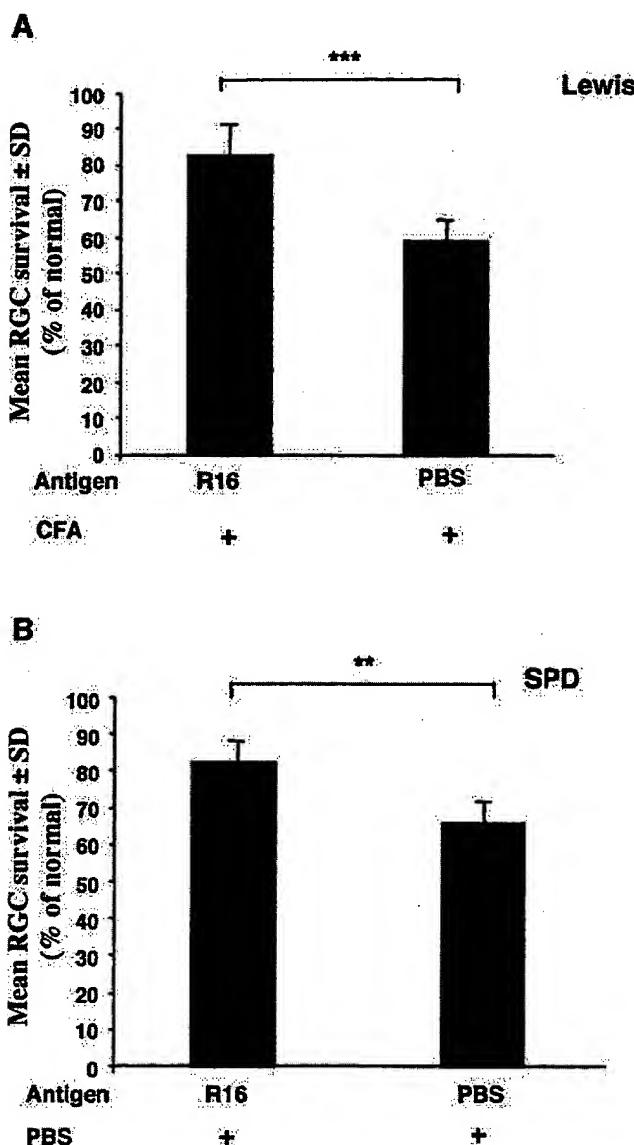


FIGURE 3. Immunization of Lewis or SPD rats with the uveitogenic peptide R16, immediately after the first increase in IOP, protected RGCs from death caused by an increase in IOP. Adult (A) Lewis or (B) SPD rats were immunized with R16 emulsified in CFA. Control rats were injected with PBS in CFA. Three weeks after the first increase in IOP the retinas were stained. They were excised 24 hours later, and survival of RGCs was calculated as described in Figure 1. In Lewis rats, significantly more RGCs survived in the group vaccinated with R16 than in the PBS-injected group ($***P < 0.0001$). A similar pattern was observed in the SPD rats, in which significantly more RGCs survived after vaccination with R16 ($n = 6$) than after injection with PBS ($n = 4$; $**P < 0.003$). As the control, normal retinas of both strains ($n = 3$ for each strain) were labeled at the same time, and their RGCs were counted and taken as 100%. In the SPD rats, clinical disease did not develop. The presented data are from one of four (A) and one of two (B) independent experiments with similar results.

the R16-vaccinated Lewis rats (Fig. 4B). According to the results, it seemed that although inflammation had some destructive effect on the healthy retina, its beneficial effect on the IOP-damaged retina outweighed the cost. In light of these findings and those in our previous work^{15,32,33} showing that immune activity is important in promoting survival of damaged neurons, we were interested in finding out whether a high dose of steroids, often used clinically in acute CNS insults to

wipe out inflammation, would be destructive to RGCs. Examination of the effect of MP on RGC survival in R16-vaccinated animals, as well as in nonvaccinated rats or control rats immunized with PBS in CFA, showed that MP prevented the R16-induced development of clinical EAU in Lewis rats (manifested by the low uveitis scores obtained by these rats compared with R16-vaccinated rats that were not treated with MP). However, in these EAU-susceptible rats, MP caused some loss of RGCs beyond the weak loss caused by R16-induced inflammation (Fig. 5). Thus, MP did not protect RGCs from inflammation-induced death. Moreover, injection of MP into Lewis rats after they were injected with PBS in CFA showed that that the MP itself caused RGC death; the number of surviving RGCs in this group (1630 ± 285 , $n = 10$) was even smaller (though not significantly) than that in the Lewis rats treated with MP after vaccination with R16 in CFA (1830 ± 352 , $n = 9$; $P = 0.19$). In a separate set of experiments, using the same groups of rats ($n = 5$ –10 in each group), we also compared the effect, in

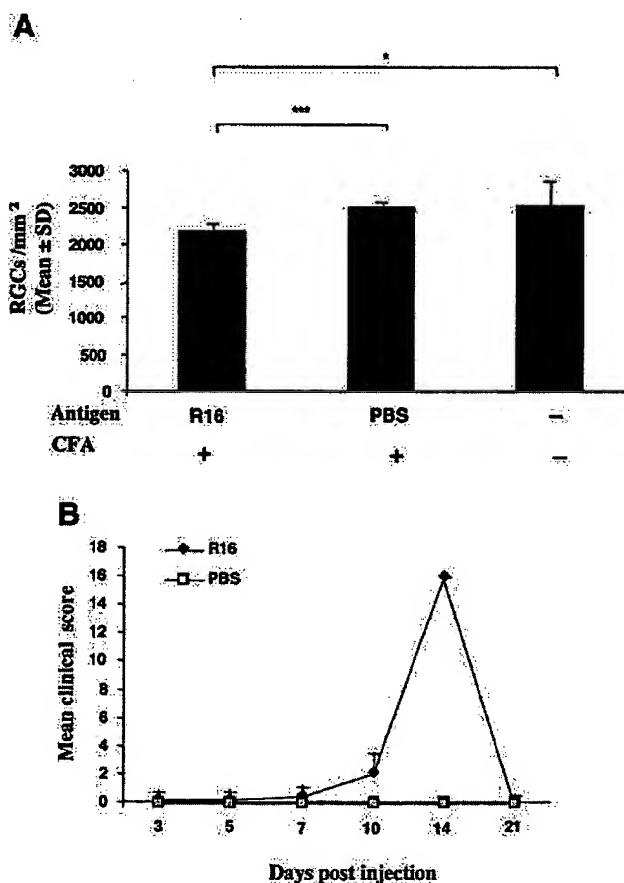


FIGURE 4. Experimental autoimmune uveitis (EAU) caused some death of RGCs. (A) Lewis rats were immunized with R16 emulsified in CFA, and control Lewis rats were injected with PBS in CFA ($n = 6$ in each group). RGC survival was measured by retrograde labeling with rhodamine dextran 3 weeks after immunization (by which time the disease had resolved itself). Immunization with R16 caused a small but significant loss of RGCs. The average number of RGCs per field in the R16-treated group was significantly lower than that in the group injected with PBS in CFA ($***P < 0.001$) or in the normal group ($*P < 0.05$). The difference between the two control groups was not significant ($P = 0.87$). (B) Mean clinical scores for EAU in Lewis rats injected with R16 (see grading in Table 1). The first signs appeared on day 10 after R16 immunization, peak symptoms were seen on day 14, and the disease resolved itself by day 21. Clinical disease did not develop in control rats injected with PBS. The presented data are from one of four independent experiments with similar results.

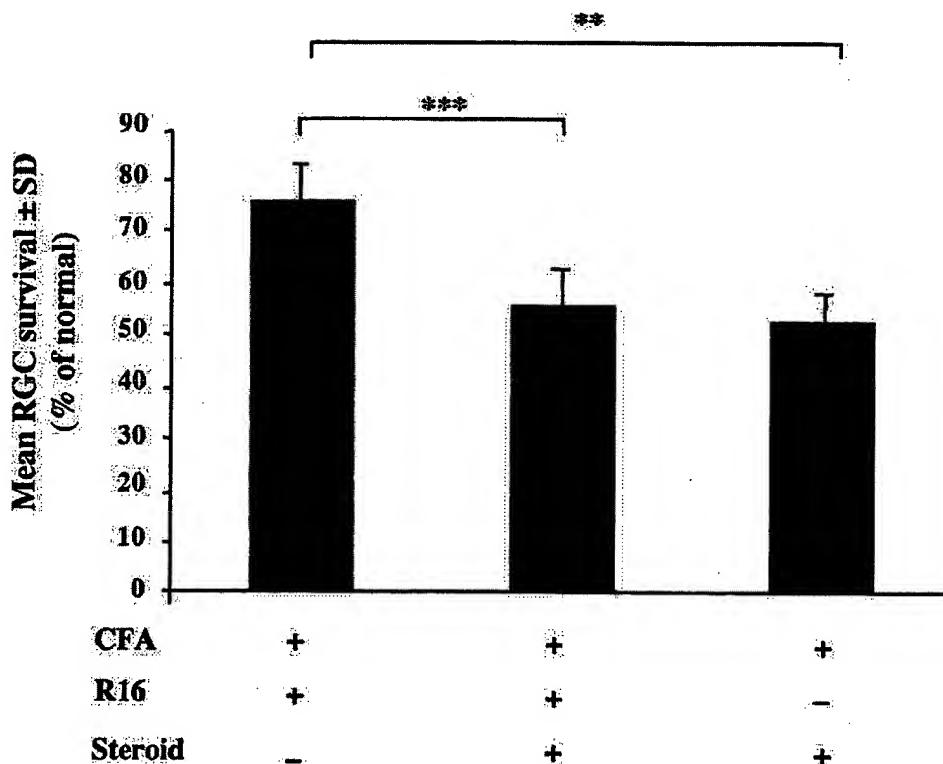


FIGURE 5. Steroid treatment alleviates EAU symptoms but reduces the number of viable RGCs in Lewis rats with EAU. Adult Lewis rats were vaccinated with R16 emulsified in CFA or injected IP immediately afterward with 30 mg/kg of MP (125 mg/2 mL). The MP injection was repeated 3, 6, 9, and 12 days after the start of the experiment. After 3 weeks, the retinas were stained and 24 hours later were excised and the RGCs counted. The number of surviving RGCs in R16-vaccinated rats treated with MP ($n = 9$) was significantly smaller than in R16-vaccinated rats not treated with MP ($n = 9$; $***P < 0.0001$). In addition, RGC survival in the group injected with PBS in CFA and MP ($n = 10$) was significantly lower than that in the group injected with PBS in CFA only ($n = 4$; $**P < 0.01$). The presented data are from one of two independent experiments with similar results.

healthy Lewis rats, of MP injection with the effect of injection of PBS in CFA. The number of surviving RGCs per square millimeter in naïve Lewis rats injected with MP was 1815 ± 224 ($n = 5$) compared with 2715 ± 128 ($n = 9$) in naïve Lewis rats that were not injected ($P < 0.01$). No increase in IOP was observed in any of the Lewis rats after R16 vaccination or MP treatment (Table 2), ruling out the possibility that the RGC death attributed to the inflammation or to the steroid treatment was actually caused by an increase in IOP.

In further experiments, we also immunized naïve SPD rats with R16 emulsified in CFA. As expected, no clinical disease or RGC loss was observed. This finding prompted us to examine the effect of MP in rats in which EAU does not develop. Injection of MP alone in healthy SPD rats resulted in RGC loss, but the loss was reduced when these rats were also vaccinated

with R16 (the RGC count in the nonvaccinated MP-treated group ($n = 5$) was 1341 ± 110 cells/mm², compared with 1668 ± 129 cells/mm² in the vaccinated group ($n = 4$), $P < 0.01$; Fig. 6). These results indicate that R16, unlike MP, does not cause RGC loss in an EAU-resistant strain. Moreover, R16 was partially capable of protecting the RGCs of SPD rats from MP-induced death. It thus appears that autoimmunity provides some protection against MP-induced death in SPD rats.

DISCUSSION

In this study, using a rat model for IOP-associated chronic glaucoma, we show that autoimmunity protects RGCs from death induced by high IOP. The protective autoimmune T cells were found to be specific to antigens residing in the eye, rather than to myelin-associated antigens found in the nerve fibers.

Previous studies in our laboratory have demonstrated that T cells directed against myelin antigens protect neurons from the consequences of axonal injury.⁹ Thus, passive transfer of T cells directed against myelin-derived peptides or active vaccination with those peptides reduced RGC loss after optic nerve injury.^{15,34} It was further discovered that the ability to benefit from T cells directed against myelin antigens is not merely a reflection of experimental manipulation, but is the body's natural way of coping with the injury. The physiological autoimmune response was found to be amenable to boosting.^{3,20}

Our group discovered, in addition, that a T-cell-mediated protective mechanism is also operative when the damage is caused by a direct injection of glutamate into the vitreous.^{2,3} In that case however, myelin antigens failed to boost the response, whereas after an intraocular injection of glutamate, antigens residing in the eye evoked an immune response that successfully reduced the loss of RGCs.⁹ It thus appears that for the T cells to be effective, they should be activated at the site of the stress where the relevant antigens are presented to them.⁹

TABLE 2. IOPs in Lewis and SPD Rats, with and without R16 Vaccination and/or Steroids

Group (n)	Mean IOP ± SD
Normal Lewis	17.78 ± 1.08
Normal SPD	17.13 ± 1.28
Lewis+R16	19.83 ± 1.54
Lewis+steroids	18.8 ± 1.86
Lewis+R16+steroids	16.85 ± 2.25
SPD+steroids	18.04 ± 1.32
SPD+R16+steroids	17.87 ± 1.14

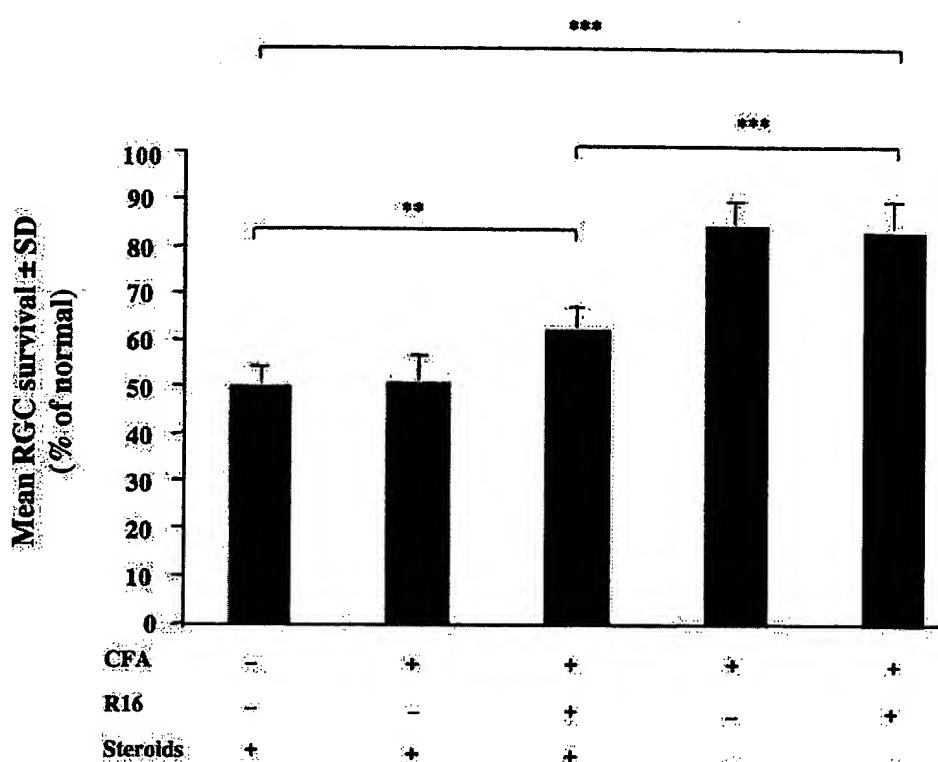
IOPs in the two tested rat strains, with or without steroid treatment, did not exceed normal values and did not differ significantly from one another. Rats were injected intraperitoneally with acepromazine (10 mg/mL) to induce a tranquil state, and 5 minutes later the IOP in both eyes was measured with a handheld tonometer after application of 0.4% benoxinate to the cornea. The average of 10 measurements obtained from each eye was recorded. Results are the mean ± SD. No significant differences were observed between any of the tested groups. Normal Lewis, $n = 7$; all other groups, $n = 6$.

FIGURE 6. R16 vaccination protected RGCs from steroid-induced death in a strain that is resistant to development of EAU. SPD rats (EAU-resistant) were vaccinated with R16 or injected with PBS and treated with MP according to the same protocol as that used for Lewis rats (described in Fig. 4). Three weeks later, the retinas were stained and excised, and the numbers of surviving RGCs were determined. The percentage of surviving RGCs was significantly smaller in the nonvaccinated group of rats injected every other day with MP ($n = 5$) than in the group vaccinated with R16 and not treated with MP ($n = 12$; *** $P < 0.0001$). These results show that steroid treatment can have a deleterious effect on neuronal survival. R16 immunization showed some protection of the RGCs from MP-induced RGC loss. Significantly fewer RGCs survived in rats treated only with MP than in rats treated with both R16 vaccination and MP ($n = 4$; ** $P < 0.01$). The presented data are from one of two independent experiments with similar results.

Glaucoma belongs to a group of diseases often characterized by an increase in IOP.^{35,36} In a previous study, using a rat model of IOP-induced RGC death, we showed that the immune system plays a key role in an animal's ability to resist the damaging consequences of an increase in IOP.¹⁶ Thus, rats deprived of T cells were found to be more prone to IOP-induced RGC loss than rats with normal T cell populations. In the present study we show that the ability of rats to resist IOP-induced RGC death can be improved by vaccination with antigens residing in the eye (but not in the optic nerve). The antigen found to protect RGCs against IOP-induced death in the present study is R16, a uveitis-associated peptide.^{29,31,37-39}

The present finding that protection of RGCs against IOP-induced death can be boosted by an immune response that is directed against proteins residing in the eye but not in the nerve, coupled with our previous finding that myelin proteins can elicit an immune response that protects RGCs against death induced by an axonal injury but not against death induced directly within the eye by glutamate, suggest that, at least at the early stage, IOP-induced death initiates degeneration in the eye, but not in the optic nerve head. The finding that vaccination with Cop-1 can counteract insults to RGCs imposed both by axonal injury and by toxic glutamate injection into the eye suggests that Cop-1 circumvents the tissue-specificity barrier, and is in line with the observed effect of Cop-1 in reducing IOP-induced RGC death. It is important to note that none of the therapeutic vaccinations appeared to be effective in rats deprived of mature T cells as adults (Bakalash et al., unpublished data, 2003). We have data suggesting that the beneficial effect of vaccination is manifested not only in the number of surviving RGCs, but also in functional activity, as indicated by reduction in loss of the visual evoked potential response (Ben-Shlomo et al., unpublished data, 2003).

The finding that IOP-induced RGC death could be significantly reduced by eye-derived peptides suggests that the T cells exerting a beneficial effect are those activated by antigens presented to them in the eye. It should be emphasized that the eye-derived self-peptides that were tested in this study, and



which in EAU-susceptible strains are potentially pathogenic to the eye, are not the peptides that would be selected for development of a therapy. They are useful, however, as a tool for gaining insight into the specificity of the body's physiological immune remedy against stressful conditions associated with high IOP. According to our view, under stressful conditions the immune system is harnessed by the tissue to help remove endogenous harmful substances released as a result of the stress (Shaked et al., unpublished data, 2003). The present findings indicate that when the stress is caused by high IOP, such substances reside in the eye, at least in the early stages of the disease.

It is important to note that the uveitogenic peptide that protects RGCs from IOP-induced death in naive Lewis rats caused some RGC loss, which in the long run was outweighed by its benefit. In SPD rats there was no price to pay for the benefit, as this strain is not susceptible to EAU.

Recent studies in our laboratory have shown that the phenotype of the T cells required for neuroprotection is Th1.⁴⁰ In principle, therefore, any Th1 cells that are directed against antigens presented at the site of stress can lead to neuroprotection. Such T cells, once activated, provide a source of cytokines and possibly also of neurotrophins.^{41,42} According to this view, it could be argued that the protection within the eye is associated with local inflammation controlled by autoimmunity. The specificity of the T-cell response—an anti-self response—has the role of amplifying and regulating the inflammation where it is needed. Once the cells become activated by antigens presented to them at the lesion site, the effect is nonspecific and is mediated by cytokines and other immune-derived factors, including neurotrophic factors.^{34,41,43} Transient accumulation of T cells has indeed been observed in eyes of Cop-1-vaccinated rats with high IOP (Bakalash et al., unpublished data, 2003).

One of the cytokines likely to play a role in protection is IFN- γ , a cytokine characteristic of Th1 cells.⁴⁰ Studies in vitro have shown that IFN- γ can activate microglia, which we view as stand-by cells ready to participate in the dialog between the

T cells and the neural tissue when needed and to remove deleterious matter, including glutamate toxicity, from the site of stress (Shaked et al., unpublished data, 2003).

In Lewis rats with EAU, treatment with a high dose of the steroid drug MP attenuated the symptoms of inflammation. However, the treatment resulted in the death of more RGCs than was caused by the autoimmune disease itself. In naïve SPD rats, treatment with MP also resulted in a significant loss of RGCs, which was, however, slightly attenuated by the induction of an autoimmune response (i.e., by immunization with the self-reactive peptide R16). High-dose steroids are often used to treat patients after an acute traumatic CNS injury such as spinal cord injury. In the present study we used a high dose of steroid, not as a way to simulate therapy in an autoimmune disease like uveitis, but to highlight the paradox that autoimmunity is a defense mechanism that can be protective even against damage caused by steroids. It thus seems that autoimmunity can be viewed as a mechanism for maintenance and protection, whose absence might be more harmful than its presence. The results of this study further support our contention that epitopes of immunodominant proteins in a specific tissue are the ones selected by the tissue for maintenance, and that (in susceptible animals) development of an autoimmune disease in that tissue represents an extreme situation in which the mechanism that controls the immune response to these self-epitopes is defective.

It was recently suggested that a correlation exists between normal-tension glaucoma and autoimmune disease.⁴⁴ The present findings suggest that autoimmune disease, as opposed to an autoimmune response, can cause RGC loss. Accordingly, in cases in which normal-tension glaucoma is an outcome of autoimmune disease, the common practice of nonspecific immunosuppression by treatment with steroids should be critically reassessed. Moreover, after CNS injuries the potential benefit of steroid treatment to the eye is overridden by the negative effect to the RGCs, and perhaps also to other parts of the eye.^{26,45,46}

These findings support the development of vaccines for neuroprotection and immunomodulation designed to harness safely the body's own repair mechanism, thereby serving the dual goal of avoiding neuronal loss due to autoimmune disease and boosting autoimmunity for protection against neuronal loss caused by nonimmune risk factors.⁴⁷ Safe modulation can apparently be achieved by using a weak antigen, such as Cop-1, which can cross-react with self-reacting T cells without activating potentially pathogenic T cells.¹⁰

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References

- Schwartz M. Autoimmune involvement in CNS trauma is beneficial if well controlled. *Prog Brain Res.* 2000;128:259–263.
- Schori H, Kipnis J, Yoles E, et al. Vaccination for protection of retinal ganglion cells against death from glutamate cytotoxicity and ocular hypertension: implications for glaucoma. *Proc Natl Acad Sci USA.* 2001;98:3398–3403.
- Kipnis J, Yoles E, Schori H, et al. Neuronal survival after CNS insult is determined by a genetically encoded autoimmune response. *J Neurosci.* 2001;21:4564–4571.
- Yoles E, Schwartz M. Degeneration of spared axons following partial white matter lesion: implications for optic nerve neuropathies. *Exp Neurol.* 1998;153:1–7.
- Schwartz M, Kipnis J. Multiple sclerosis as a by-product of the failure to sustain protective autoimmunity: a paradigm shift. *The Neuroscientist.* 2002;8:405–413.
- Kipnis J, Mizrahi T, Hauben E, et al. Neuroprotective autoimmunity: Naturally occurring CD4+CD25+ regulatory T cells suppress the ability to withstand injury to the central nervous system. *Proc Natl Acad Sci USA.* 2002;99:15620–15625.
- Schwartz M. T cell mediated neuroprotection is a physiological response to central nervous system insults. *J Mol Med.* 2001;78:594–597.
- Hauben E, Ibarra A, Mizrahi T, et al. Vaccination with a Nogo-A-derived peptide after incomplete spinal-cord injury promotes recovery via a T-cell-mediated neuroprotective response: comparison with other myelin antigens. *Proc Natl Acad Sci USA.* 2001;98:15173–15178.
- Mizrahi T, Hauben E, Schwartz M. The tissue-specific self-pathogen is the protective self-antigen: The case of uveitis. *J Immunol.* 2002;169:5971–5977.
- Kipnis J, Schwartz M. Dual action of glatiramer acetate (Cop-1) as a treatment for autoimmune diseases and a vaccine for protective autoimmunity after CNS injury. *Trends Mol Med.* 2002;8:319–323.
- Nevo U, Kipnis J, Golding I, et al. Autoimmunity as a special case of immunity: removing threats from within. *Trends Mol Med.* 2003;9:88–93.
- Kipnis J, Yoles E, Porat Z, et al. T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies. *Proc Natl Acad Sci USA.* 2000;97:7446–7451.
- Hafler DA, Weiner HL. Antigen specific therapies for the treatment of autoimmune diseases. *Agents Actions Suppl.* 1995;47:59–77.
- Hauben E, Butovsky O, Nevo U, et al. Passive or active immunization with myelin basic protein promotes recovery from spinal cord contusion. *J Neurosci.* 2000;20:6421–6430.
- Fisher J, Levkovich-Verbin H, Schori H, et al. Vaccination for neuroprotection in the mouse optic nerve: implications for optic neuropathies. *J Neurosci.* 2001;21:136–142.
- Bakalash S, Kipnis J, Yoles E, Schwartz M. Resistance of retinal ganglion cells to an increase in intraocular pressure is immune-dependent. *Invest Ophthalmol Vis Sci.* 2002;43:2648–2653.
- Jia L, Cepurna WO, Johnson EC, Morrison JC. Patterns of intraocular pressure elevation after aqueous humor outflow obstruction in rats. *Invest Ophthalmol Vis Sci.* 2000;41:1380–1385.
- Levkovich-Verbin H, Quigley HA, Martin KR, et al. Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. *Invest Ophthalmol Vis Sci.* 2002;43:402–410.
- Jia L, Cepurna WO, Johnson EC, Morrison JC. Effect of general anesthetics on IOP in rats with experimental aqueous outflow obstruction. *Invest Ophthalmol Vis Sci.* 2000;41:3415–3419.
- Yoles E, Hauben E, Palgi O, et al. Protective autoimmunity is a physiological response to CNS trauma. *J Neurosci.* 2001;21:3740–3748.
- Brocke S, Gijbels K, Allegretta M, et al. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature.* 1996;379:343–346.
- Hauben E, Agranov E, Gothilf A, et al. Posttraumatic therapeutic vaccination with modified myelin self-antigen prevents complete paralysis while avoiding autoimmune disease. *J Clin Invest.* 2001;108:591–599.
- Chen MS, Huber AB, van der Haar ME, et al. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature.* 2000;403:434–439.
- Merkler D, Metz GA, Raineteau O, et al. Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-A. *J Neurosci.* 2001;21:3665–3673.
- Huber AB, Schwab ME. Nogo-A, a potent inhibitor of neurite outgrowth and regeneration. *Biol Chem.* 2000;381:407–419.
- Steinsapir KD, Goldberg RA, Sinha S, Hovda DA. Methylprednisolone exacerbates axonal loss following optic nerve trauma in rats. *Restor Neurol Neurosci.* 2000;17:157–163.
- Derendorf H, Mollmann H, Rohdewald P, Rehder J, Schmidt EW. Kinetics of methylprednisolone and its hemisuccinate ester. *Clin Pharmacol Ther.* 1985;37:502–507.
- Dua HS, Abrams MS, Barrett JA, Donoso LA. The effect of retinal autoantigens and their peptides on the inhibition of experimental autoimmune uveitis. *Eye.* 1992;6:447–452.

29. Wacker WB, Kalsow CM. Autoimmune uveo-retinitis in the rat sensitized with retina photoreceptor cell antigen. *Int Arch Allergy Appl Immunol.* 1973;45:582-592.
30. Gregerson DS, Obritsch WF, Fling SP, Cameron JD. S-antigen-specific rat T cell lines recognize peptide fragments of S-antigen and mediate experimental autoimmune uveoretinitis and pinealitis. *J Immunol.* 1986;136:2875-2882.
31. Caspi RR. Immune mechanisms in uveitis. *Springer Semin Immunopathol.* 1999;21:113-124.
32. Schori H, Yoles E, Schwartz M. T-cell-based immunity counteracts the potential toxicity of glutamate in the central nervous system. *J Neuroimmunol.* 2001;119:199-204.
33. Schori H, Lantner F, Shachar I, Schwartz M. Severe immunodeficiency has opposite effects on neuronal survival in glutamate-susceptible and -resistant mice: adverse effect of B cells. *J Immunol.* 2002;169:2861-2865.
34. Moalem G, Leibowitz-Amit R, Yoles E, et al. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat Med.* 1999;5:49-55.
35. Bathija R, Gupta N, Zangwill L, Weinreb RN. Changing definition of glaucoma. *J Glaucoma.* 1998;7:165-169.
36. Quigley HA. Neuronal death in glaucoma. *Prog Retinal Eye Res.* 1999;18:39-57.
37. Doeke G, van der Gaag R, Rothova A, et al. Humoral and cellular immune responsiveness to human S-antigen in uveitis. *Curr Eye Res.* 1987;6:909-919.
38. Wacker WB. Experimental allergic uveitis. Studies on characterization and isolation of the pathogenic retina antigen *Int Arch Allergy Appl Immunol.* 1973;45:639-656.
39. Wacker WB. Proctor Lecture. Experimental allergic uveitis Investigations of retinal autoimmunity and the immunopathologic responses evoked. *Invest Ophthalmol Vis Sci.* 1991;32:3119-3128.
40. Kipnis J, Mizrahi T, Yoles E, Ben-Nun A, Schwartz M. Myelin specific Th1 cells are necessary for post-traumatic protective autoimmunity. *J Neuroimmunol.* 2002;130:78-85.
41. Barouch R, Schwartz M. Autoreactive T cells induce neurotrophin production by immune and neural cells in injured rat optic nerve: implications for protective autoimmunity. *FASEB J.* 2002;16:1304-1306.
42. Moalem G, Gdalyahu A, Shani Y, et al. Production of neurotrophins by activated T cells: implications for neuroprotective autoimmunity. *J Autoimmun.* 2000;15:331-345.
43. Kerchensteiner M, Gallmeier E, Behrens L, et al. Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *J Exp Med.* 1999;189:865-870.
44. Wax MB. Is there a role for the immune system in glaucomatous optic neuropathy? *Curr Opin Ophthalmol.* 2000;11:145-150.
45. Steiner I, Brenner T, Mizrachi-Kol R, Abramsky O. Development of experimental allergic encephalomyelitis during steroid administration: outcome of neurological immune-mediated disorders under immunosuppressive therapy. *Isr J Med Sci.* 1991;27:365-368.
46. Schmidt J, Gold R, Schonrock L, et al. T-cell apoptosis in situ in experimental autoimmune encephalomyelitis following methylprednisolone pulse therapy. *Brain.* 2000;123:1431-1441.
47. Schwartz M, Yoles E. Self-destructive and self-protective processes in the damaged optic nerve: implications for glaucoma. *Invest Ophthalmol Vis Sci.* 2000;41:349-351.

Therapeutic vaccine for acute and chronic motor neuron diseases: Implications for amyotrophic lateral sclerosis

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Therapeutic vaccination with Copaxone (glatiramer acetate, Cop-1) protects motor neurons against acute and chronic degenerative conditions. In acute degeneration after facial nerve axotomy, the number of surviving motor neurons was almost two times higher in Cop-1-vaccinated mice than in nonvaccinated mice, or in mice injected with PBS emulsified in complete Freund's adjuvant ($P < 0.05$). In mice that express the mutant human gene Cu/Zn superoxide dismutase G93A (SOD1), and therefore simulate the chronic human motor neuron disease amyotrophic lateral sclerosis, Cop-1 vaccination prolonged life span compared to untreated matched controls, from 211 ± 7 days ($n = 15$) to 263 ± 8 days ($n = 14$; $P < 0.0001$). Our studies show that vaccination significantly improved motor activity. In line with the experimentally based concept of protective autoimmunity, these findings suggest that Cop-1 vaccination boosts the local immune response needed to combat destructive self-compounds associated with motor neuron death. Its differential action in CNS autoimmune diseases and neurodegenerative disorders, depending on the regimen used, allows its use as a therapy for either condition. Daily administration of Cop-1 is an approved treatment for multiple sclerosis. The protocol for non-autoimmune neurodegenerative diseases such as amyotrophic lateral sclerosis, remains to be established by future studies.

Amyotrophic lateral sclerosis (ALS) is a progressive disease of the upper and lower motor neurons, in most cases causing death by respiratory failure. Its etiology, prognosis, and progression have been intensively studied over the last decade. Two etiological factors have so far been identified: mutations in the Cu/Zn superoxide dismutase (SOD) gene on chromosome 21 (1) and in the alsin gene putatively encoding a ras GTPase (2, 3). These mutations, however, account for fewer than 10% of patients with ALS. Many factors that contribute to the pathogenesis of ALS are common to other chronic degenerative disorders of the central nervous system (CNS), such as oxidative stress, excitotoxicity, deprivation of trophic support, and ionic imbalance (4). The pathogenesis of chronic selective death of anterior horn cells can be studied in transgenic mice expressing the mutant human Cu/Zn SOD G93A (SOD1) gene (5, 6).

There have been numerous clinical and experimental attempts to halt the progression of ALS by blocking different mediators of cytotoxicity (7). Because not all ALS patients have defective genes, the results of such attempts are often verified by studying motor neuron death (common to all cases of ALS) in an animal model of acute peripheral nerve axotomy (8, 9). The only drug currently used to slow down the progression of ALS, although with only modest effect, is riluzole, a putative blocker of glutamate release (10, 11).

The immune system, which protects the organism from the effects of invasion by pathogenic microorganisms, was recently found to be protective against destructive self-components as well (12–16). In acute neurodegenerative conditions caused by mechanical (e.g., crush injury or axotomy) (12, 14) or biochemical insults (e.g., glutamate or oxidative stress) (15), more neurons survive

in the presence of an evoked anti-self T cell-mediated response than in its absence, provided that the evoked response is well regulated (13, 16–19). The protective T cell-mediated response can be boosted, without risk of autoimmune disease induction, by administration of copolymer-1 (Cop-1; Copaxone), a synthetic polypeptide consisting of the amino acids tyrosine, glutamate, alanine, and lysine (15, 20). It was recently suggested that this compound can activate a wide range of self-reactive T cells (20, 21). In a model of a chronic neurodegenerative disorder associated with optic nerve neuropathy, such as glaucoma, Cop-1 vaccination was found to bypass the tissue-specific barrier imposed by antigens residing in the damaged tissue (15, 22) and to significantly increase neuronal survival (15, 20).

Cop-1 is a Food and Drug Administration-approved drug for the treatment of multiple sclerosis (MS). In this study, by treating mice with Cop-1 (according to a different regimen from that used for MS), we show that motor neurons can be protected against both acute and chronic degeneration.

Materials and Methods

Acute Motor Neuron Disorder. Adult female mice (12 weeks old, 20–25 g) of the C57BL/6JO1aHsd strain (Harlan Winkelmann, Borchen, Germany) were subjected to unilateral facial nerve axotomy. Mice in the experimental group received a total of 100 µg of Cop-1 in complete Freund's adjuvant (CFA). Control animals were axotomized and were either untreated or injected with PBS emulsified in CFA. Seven days later a facial-facial anastomosis was created in anesthetized mice (100 mg of Ketaset plus 5 mg of Rompun per kg of body weight) by microsurgical reconnection of the proximal stump to the distal stump with two 11-0 epineurial sutures (Ethicon EH 7438G, Norderstedt, Germany). The wound was closed with three 4-0 skin sutures. For assessment of recovery, facial motor neurons supplying the whiskerpad muscles were retrogradely labeled by injection of 30 µl of 1% aqueous solution of the fluorescent retrograde tracer FluoroGold plus 2% dimethyl sulfoxide (DMSO) injected into the muscles of each whisker pad. Seven days later, the mice were re-anesthetized and perfused transcardially with 0.9% NaCl followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 20 min. The brains were removed and 50-µm-thick coronal sections were cut through the brain stems with a vibratome. Sections were observed with a Zeiss Axioskop 50 epifluorescence microscope through a custom-made HQ-Schmalband-filter set for FluoroGold (AHF Analysentechnik, Tübingen, Germany).

Abbreviations: ALS, amyotrophic lateral sclerosis; SOD, superoxide dismutase; MS, multiple sclerosis; RGC, retinal ganglion cell; CFA, complete Freund's adjuvant.

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Quantitative Analysis. For image analysis, a charged coupled device video camera system (Optronics Engineering Model DEI-470, Goleta, CA) combined with the image analyzing software Optimas 6.5 (Optimas, Bothell, WA) was used to manually count the retrogradely labeled facial motor neurons on the computer screen (23). Employing the fractionator principle (24), all retrogradely labeled motor neurons with visible cell nuclei were counted in every second section of the 50- μ m-thick sections through the facial nucleus on both the operated and the unoperated side. Counting was done by two observers who were blinded to the treatment received by the rats.

Electrophysiological Assessment. The two large hairs of the C-row on each side of the face were used for biometric analysis. With the mice under light ether narcosis, all other vibrissae were clipped with small fine scissors. A digital camcorder (Panasonic NV DX-110 EG) was used to videotape the actively exploring mice for 3–5 min. After calibration, video images of whisking behavior were sampled at 50 Hz (50 fields per sec), with the video camera shutter opened for 4 msec. Images were recorded on AY-DVM 60 EK minicassettes. The video sequences were slowly reviewed and 1.5-sec sequence fragments from each mouse were selected for analysis of whisking biometrics. The selection criteria used were stable position of the head, frequency of whisking, and degree of vibrissal protraction. The selected sequences were captured by a 2D/Manual Advanced Video System PEAK Motus 2000 (PEAK Performance Technologies, Englewood, CO). The spatial model consisted of three reference points (tip of the nose and the inner angles of both eyes). Each vibrissa is represented in the spatial model by two points: its base and a point on the shaft 0.5 cm from the base.

Glutamate Injection. With the aid of a binocular microscope, the right eye of the anesthetized mouse was punctured in the upper part of the sclera with a 27-gauge needle, and a 10- μ l Hamilton syringe with a 30-gauge needle was inserted as far as the vitreal body. Mice were injected with L-glutamate (200 nmoles) (Sigma) dissolved in saline (total volume 1 μ l).

Labeling of Retinal Ganglion Cells. Mice were anaesthetized as described above and placed in a stereotactic device. The skull was exposed, and the bregma was identified and marked. The site selected for injection was in the superior colliculus, 2.92 mm posterior to the bregma, 0.5 mm lateral to the midline, and at a depth of 2 mm from the brain surface. A window was drilled in the scalp above the designated coordinates in the right and left hemispheres. The neurotracer dye FluoroGold (5% solution in saline, Fluorochrome, Denver) was stereotactically applied (1 μ l, at a rate of 0.5 μ l/min in each hemisphere) by using a Hamilton syringe, and the skin over the wound was sutured.

Assessment of Retinal Ganglion Cell (RGC) Survival. At the end of the experimental period, the mice were killed by injection of a lethal dose of pentobarbitone (170 mg/kg). Their eyes were enucleated and the retinas were detached and prepared as flattened whole mounts in 4% paraformaldehyde in PBS. Labeled cells from four to six fields of identical size (0.076 mm²) were counted. The counted fields were located at approximately the same distance from the optic disk (0.3 mm) to allow for variations in RGC density as a function of distance from the optic disk. Fields were counted under the fluorescence microscope (magnification, $\times 800$) by observers blinded to the treatment received by the mice. The average number of RGCs per field was calculated for each retina. The number of RGCs in the contralateral (uninjured) eye was also counted, and served as an internal control.

ALS Model. Transgenic mice [B6SJL-TgN (SOD1-G93A) 1Gur, supplied by The Jackson Laboratory], aged 60 days, were vaccinated with Cop-1 (75 μ g) emulsified in complete Freund's adjuvant

(CFA; Difco) containing 5 mg/ml *Mycobacterium tuberculosis*. The emulsion (total volume 200 μ l) was injected into the hind foot pad, and thereafter the mice were treated daily with oral Cop-1 (12.5 mg/kg/day) given in the drinking water. Their motor activity and mortality were monitored. The transgene in these mice carries a mutant human SOD1 allele containing the Gly-93 \rightarrow Ala (G93A) gene. Paralysis is caused by the progressive loss of motor neurons from the spinal cord. Control mice were either left untreated or received 30 mg/kg riluzole daily. A second group of transgenic mice, expressing more copies of the defective SOD1 mutants, was treated by vaccination with Cop-1 mixed with Alum-Phos. This was administered either as two injections given a week apart or as three injections. The first injection was given when the mice were \approx 60–70 days old, the second injection was given a week later, and the third injection was given a month later. Control groups received either a single injection or two injections of Alum-Phos.

The mice were allowed to grasp and hold onto a vertical wire (2 mm diameter) with a small loop at the lower end. Their activity was recorded individually by a computerized system and assessed daily. For statistical evaluation, the rotarod activity was normalized to the mean activity of each mouse from day 40 to day 60.

Results

Cop-1 Vaccination Protects Against Motor Neuron Death Induced by Acute Facial Nerve Axotomy. Transection of the facial nerve in the adult mouse is known to cause an easily visible late degeneration of 20–35% of the axotomized motor neurons (25). Mice were immunized with Cop-1 ($n = 10$) or injected with PBS ($n = 9$), both emulsified in CFA, and 7 days later were subjected to facial nerve axotomy. Mice in a third group ($n = 8$) were axotomized without prior immunization, and mice in a fourth group ($n = 7$) were left intact. Eight weeks after axotomy, as shown in Fig. 1 and Table 1, the mean number of FluoroGold-labeled motor neurons in the mice vaccinated with Cop-1 was significantly larger than the number obtained in the group injected with PBS in CFA or in the untreated control group ($P < 0.05$). Immunization with PBS in CFA had no protective effect. Treatment with Cop-1 had no effect on the number of motor neurons in the unlesioned facial nucleus.

Cop-1 Administration Preserves Motor Neuron Activity After Acute Axotomy. To determine whether the larger number of motor neurons found in the Cop-1-treated axotomized mice than in the controls was associated with functional improvement, we biometrically analyzed whisking behavior. Baseline parameters of whisking behavior were documented in intact control mice. Under normal physiological conditions, the mystacial vibrissae are erect with anterior orientation. Their simultaneous sweeps, known as "whisking" or "sniffing" (26, 27), occur 5–11 times per second (28, 29). The key movements of this motor activity are the protraction and retraction of the vibrissal hairs by the piloerector muscles, which are innervated by the buccal branch of the facial nerve (30). When the facial nerve is transected, the vibrissae acquire a caudal orientation and remain motionless. We used this model (Fig. 4, which is published as supporting information on the PNAS web site, www.pnas.org) to evaluate the following parameters: (i) protraction (forward movement of the vibrissae), measured by the rostrally opened angle between the mid-sagittal plane and the hair shaft (large protractions are represented by small angle values); (ii) whisking frequency, represented by cycles of protraction and retraction (passive backward movement) per second; (iii) amplitude (the difference, in degrees, between maximal retraction and maximal protraction); (iv) angular velocity during protraction (in degrees per second); and (v) angular acceleration during protraction (in degrees per second per second).

Mice subjected to facial nerve axotomy and Cop-1 administration exhibited significantly better whisking activity than the other groups of mice, as demonstrated by the amplitude, the angular velocity

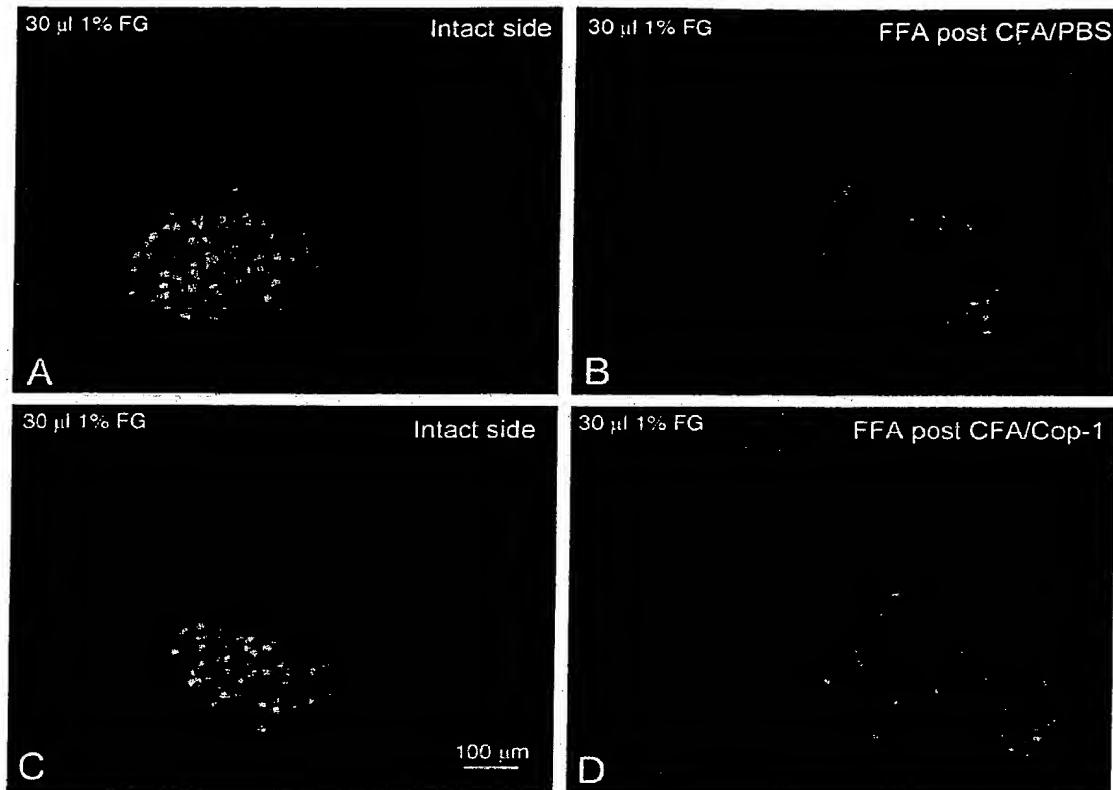


Fig. 1. Rescue of motor neurons by Cop-1 administered after facial nerve axotomy. Retrograde neuronal labeling after injection of FluoroGold into the whiskerpad showed no differences in the localization or amount of motor neurons in the intact facial nucleus between mice immunized with PBS in CFA (*A*) and mice injected with Cop-1 in CFA (*C*). In contrast, the lesioned facial nucleus in control mice pretreated with PBS in CFA contained significantly fewer labeled motor neurons than that of the lesioned facial nucleus in mice pretreated with Cop-1 in CFA (*B* vs. *D*).

during protraction, and the angular acceleration during protraction (Table 2).

The results presented above suggest that motor neurons in a mouse model of an acute degenerative disorder can benefit from protection induced by Cop-1 vaccination.

Cop-1 Treatment Increases the Life Expectancy of ALS Mice. Mice overexpressing the defective human SOD1 gene develop a motor disease that closely resembles the human disease ALS. The motor dysfunction eventually causes their death. To assess the potential efficacy of Cop-1 vaccination in a model of a chronic neurodegenerative disorder, we first determined whether the effect of a single

immunization with Cop-1 emulsified in CFA is long-lived. The selected dose was 75 μ g, because this was found to be optimally effective in a range of tested Cop-1 dosages between 25 and 225 μ g (H.S., Hila Avidan, and M.S., unpublished data). We used a mouse model of glutamate toxicity to first measure the length of time that a single vaccination with Cop-1 emulsified in CFA protects against glutamate toxicity. Mice were subjected to local toxicity of glutamate, which was injected intravitreally at different time intervals after vaccination. One week after the glutamate injection, the number of surviving neurons were counted. Fig. 2*A* shows that a single injection with Cop-1 emulsified in CFA was significantly more effective in protecting against glutamate toxicity than the injection of PBS emulsified in CFA. Significant protection against glutamate toxicity was observed in mice that had received toxic amounts of glutamate up to 60 days (but not more) after the vaccination (Fig. 2*B*). The weak effect of the adjuvant by itself (Figs. 1 and 2*A*), coupled with the persistent nature of postvaccination immune-dependent protection, encouraged us to examine the efficacy of Cop-1 immunization for chronic ALS.

Mice immunized with Cop-1 at the age of 60 days and age-matched untreated control mice were observed daily and weighed weekly. The mice received a single immunization of Cop-1 emulsified in CFA, followed by oral immunization with Cop-1 given in the drinking water. The age at symptom onset was determined as the age (in days) at the time of first appearance of tremors or shaking of the limbs, or hanging (rather than splaying out) of the hind limbs when the mouse was held in the air by the tail. Loss of the righting reflex was taken to indicate the end stage of the disease. In the absence

Table 1. Effect of Cop-1 vaccination on survival of motor neurons

Group	Unlesioned facial nucleus	Lesioned facial nucleus
A: Intact mice (<i>n</i> = 7)	1,559 \pm 135	1,707 \pm 90 ^{*B,C,D}
B: FFA only (<i>n</i> = 8)	1,434 \pm 106	670 \pm 178 ^{*A,D}
C: FFA after PBS/CFA injection (<i>n</i> = 9)	1,605 \pm 142	766 \pm 104 ^{*A,D}
D: FFA after vaccination with Cop-1 in CFA (<i>n</i> = 10)	1,640 \pm 186	1,172 \pm 152 ^{*A,B,C}

Numerical values of the results shown in Fig. 1. Data are presented as means \pm SD. Differences between the experimental groups were detected by applying a one-way analysis of variance (ANOVA) and a post hoc *t* test for unpaired data with Bonferroni-Holm's correction.

Table 2. Effect of Cop-1 vaccination on recovery of whisking behavior after facial nerve axotomy

Group	Frequency, Hz	Angle at maximal protraction, °	Amplitude, °	Angular velocity during protraction, °/sec	Angular acceleration during protraction, °/sec ²
A: Intact mice (<i>n</i> = 7)	6.0 ± 1.0	65.1 ± 22	40 ± 14 ^{B,C}	627 ± 346 ^{B,C,D}	20,084 ± 1508 ^{B,C,D}
B: FFA only (<i>n</i> = 8)	5.0 ± 2.0	81.2 ± 27	11.0 ± 6.0 ^{A,D}	75 ± 43 ^A	1,655 ± 1146 ^A
C: FFA treated with PBS/CFA (<i>n</i> = 9)	5.3 ± 1.2	64.4 ± 6.3	22.1 ± 9.9 ^{A,D}	214 ± 70 ^A	3,874 ± 889 ^A
D: FFA treated with Cop-1/CFA (<i>n</i> = 10)	5.5 ± 0.9	68.2 ± 23.05	38.9 ± 10.6 ^{B,C}	347.8 ± 87.3 ^A	6,713 ± 2071 ^A

Biometrics of normal and recovering whisking behavior in intact mice (group A) and in mice subjected to FFA only (group B), mice subjected to FFA after injection of PBS in CFA (group C), and mice subjected to FFA after injection of Cop-1 in CFA (group D). Values are means ± SD. Superscript letters indicate groups with significantly different values (*, *P* < 0.05).

of treatment, the mice in our study (*n* = 14) died at the age of 211 ± 7 days (mean ± SD). Mice immunized with Cop-1 (*n* = 15) lived for 263 ± 8 days. Thus, vaccination with Cop-1 dramatically increased the life expectancy of the ALS mice (Fig. 3A). As a positive control, 15 mice were given a daily dose (30 mg/kg) of riluzole, the only drug currently given to ALS patients. Those mice survived for 230 ± 7 days. In addition to the increase of almost 25% in life span, disease onset (manifested by motor performance) was delayed (Fig. 3B), indicating that the benefit was also expressed in the quality of life, both at preclinical and at clinical stages (Fig. 3B). Normal

values for each mouse were obtained by assessing nocturnal motor activity (from 8 p.m. to 8 a.m.) between the ages of 40 and 60 days, by using the rotarod apparatus (Laser- und Medizin-Technologie, Berlin). With the object of translating these findings into a future treatment modality for ALS patients, we tested G93A transgenic mice, which express a large number of copies of the human mutant form of SOD1, using Cop-1 emulsified in CFA or in Alum-Phos (as CFA is not suitable for human therapy), or Cop-1 without adjuvant. The

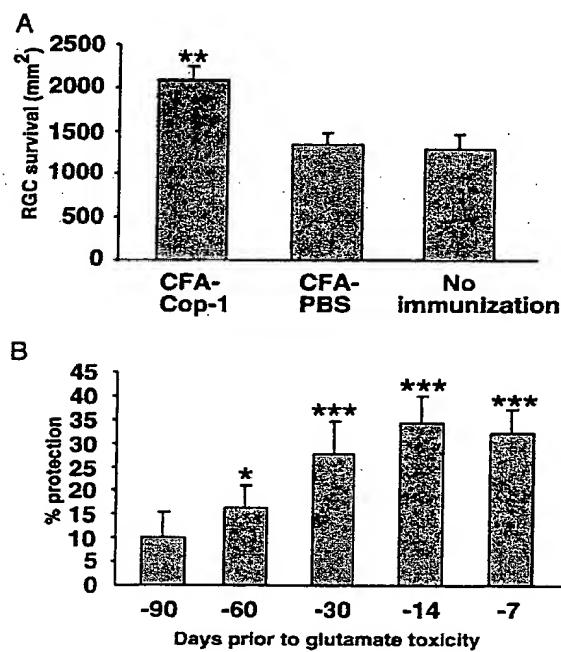


Fig. 2. Cop-1 vaccination protects RGCs against glutamate toxicity. (A) C57BL/6J mice (*n* = 6) were immunized with Cop-1 emulsified in CFA. Ten days later the mice were subjected to unilateral intraocular injection of toxic amounts of glutamate (200 nmol). As controls, we used a group of mice injected with glutamate only (*n* = 7) and a group of mice (*n* = 8) that were immunized with PBS in CFA 10 days before being exposed to glutamate. Three days after their exposure to glutamate, the RGCs were retrogradely labeled. Retinas were excised 1 week after their exposure to glutamate. RGC survival was assessed by counting the labeled cells, and is expressed as mean ± SEM per mm². A two-tailed Student's *t* test was used for statistical analysis. (B) C57BL/6J mice were immunized with Cop-1 in CFA at the indicated time points before receiving unilateral intraocular injections of toxic amounts of glutamate (200 nmol). After an additional 7 days they were killed, their retinas were excised, and RGC survival was assessed. Mice that received only glutamate were used as controls. Protection is expressed in terms of RGC survival, calculated as a percentage of RGC survival in control mice.

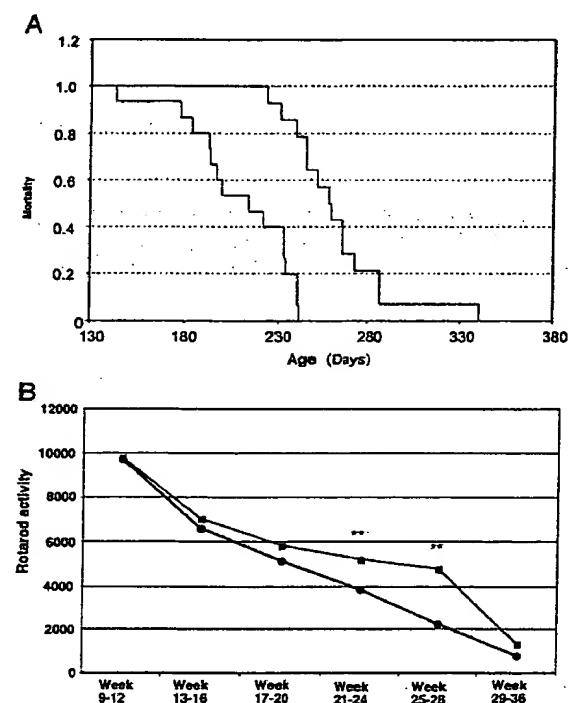


Fig. 3. Life expectancy in ALS mice immunized with Cop-1. (A) Nonvaccinated controls (*n* = 15) became paralyzed in one or more limbs and died by the age of 211 ± 7 days (mean ± SEM). Cop-1-treated mice survived for 263 ± 8 days. Survival data (expressed by mortality as a function of age in days) were analyzed by the Mantel-Cox test or Cox's proportional hazards regression analysis. Statistical significance was tested by one-way ANOVA, followed by a post hoc Student-Newman-Keuls procedure with the SPSS-PC software program (SPSS, Chicago). (B) Average rotary activity measured at the indicated time points in Cop-1-treated and untreated mice. Data are expressed as the mean ± SEM. Rotarod testing and weight were compared by ANOVA. Statistical significance was tested by one-way ANOVA followed by a post hoc Student-Newman-Keuls procedure with the SPSS-PC software program. Differences between treated and untreated mice were observed between days 12 and 20 (*P* < 0.06), between weeks 21 and 24 (*P* < 0.008), and between weeks 25 and 28 (*P* < 0.002).

optimal protocol has not yet been found. The increase in lifespan did not exceed 10% in the high-copy model (data not shown). Further studies are needed to establish the optimal dosage and regimen needed for this purpose.

Discussion

The results show that vaccination with Cop-1, in a protocol different from that used for MS patients, protects motor neurons from acute or chronic degeneration in a mouse model.

It was recently suggested that CNS insult acts as a distress signal to the immune system (13–16). Relative to normal rodents, those deprived of mature T cells lose significantly more neurons after a CNS insult (13, 15). Experimental evidence suggests that, under stress, the CNS signals to the immune system, evoking an adaptive immune response that is directed against abundant antigens residing at the site of the lesion (15, 16, 31, 32). Individuals differ in their ability to spontaneously evoke such an immune response (13). However, all individuals can benefit from boosting of the response, provided that intervention occurs at the right time, and uses the right antigen in optimal formulation so that the anti-self response is intensified, yet does not increase the risk of autoimmune disease (12, 17, 33). We further demonstrated that the same T cells (T helper 1) could apparently be responsible both for autoimmune disease and for protection from the detrimental effects of destructive self-compounds. Thus, T helper 1 cells directed against immunodominant proteins were shown to be capable of inducing autoimmune disease, as well as neuroprotection (34). Disease-free protection was achieved by inducing an immune response against cryptic epitopes residing within the same potentially pathogenic immune-abundant protein, or by using an altered pathogenic peptide to eliminate the potential pathogenicity of the peptide (17, 32).

Studies aimed at uncovering the mechanism behind this protection have revealed that T cells directed against the self-antigen migrate toward the lesion site where they become activated. Once activated, they can serve as a source of cytokines and neurotrophins. In addition, while the local neural cells clear the site of injury of cell debris and other deleterious matter (35, 36), the T cells can regulate the local innate response in which resident cells become either antigen-presenting cells or phagocytic, buffering cells (I. Shaked, O. Butovsky, T. Mizrahi, R. Gersner, X. Xiao, P. Sotropoulos, P. Tolias, R. P. Hart, and M.S., unpublished data; and ref. 37).

In an attempt to develop a method for boosting this T cell-dependent response while circumventing tissue barrier specificity and genetic susceptibility, we tested the copolymer Cop-1, an approved drug for MS. Cop-1 vaccination has been shown to protect CNS neurons against death induced by optic nerve injury or by glutamate toxicity in rat and mouse models (15, 38). In these experiments, as well as in others where vaccination was used to induce neuroprotection, we used animals immunized with PBS in CFA as controls. The protection provided by CFA was rarely significant relative to untreated controls, and was always significantly less than the optimal protection obtained by immunization with the specific antigens. In the present work too, mice treated with PBS in CFA and with PBS alone were included as control groups in the acute model of motor neuron degeneration. As expected, CFA was found to have some protective effect; however, it was significantly lower than that obtained in mice treated with Cop-1 in CFA. Recent studies have shown that Cop-1 immunization without adjuvant also leads to effective protection against glutamate toxicity (H.S., Hila Avidan, and M.S., unpublished data). This immunization method provides protection comparable to that achieved in immunization with adjuvant, but the level of T cells that can sustain the effect is retained for only two weeks. Moreover, repeated daily vaccinations with Cop-1 not only do not improve the outcome, but they even diminish the benefit derived from a single injection (H.S., Hila Avidan, and M.S., unpublished data). It seems reasonable to assume that Cop-1 immunization leading to neuroprotection, like

protective immunization with self-derived peptides, is phenotype dependent. Therefore, repeated immunization on a daily basis, even if it leads to the presence of a large number of reactive T cells, does not lead to neuroprotection if the phenotype is shifted. Recent studies showed that for the immunization with Cop-1 to be protective, IFN- γ expression by the activated lymphocytes must be sustained. This might explain why an immunization protocol that is effective in suppressing autoimmunity in an autoimmune disease such as MS is not beneficial against neurodegenerative, such as ALS, disorders where active immunity is needed (20). Thus, it appears that to develop Cop-1 for use as a therapeutic vaccine against chronic disease, it is necessary to find the formulation and the frequency of immunization. Future studies should focus on the optimal timing, frequency, and dosage of the intervention, and other adjuvants should be tested as well. In current studies, the use of Cop-1 without any vehicle is under investigation. An important finding of the present study is that the choice of animal model with regard to the level of expression of the mutant form of the human SOD1 gene might significantly influence the success of the immune intervention and perhaps also affect the protocol.

ALS is an aggressive neurodegenerative disorder in which many destructive self-components, not all of them identified, play a major role. Among the principal mediators of toxicity identified to date are glutamate and oxidative stress. The role of cellular and molecular immune factors in protecting the organism against the effects of these self-destructive agents has been debated over the years. Researchers and clinicians have also attempted to use immunosuppressants as a treatment, on the assumption that in ALS, as in many other neurodegenerative diseases, inflammation may be associated with disease propagation and therefore deleterious (39, 40). Also, the presence of anti-ganglioside antibodies in ALS patients (41) has led some researchers to suggest that ALS is an autoimmune disease. However, there is no conclusive evidence for any of these hypotheses, and therapy with immunosuppressants, including whole-body irradiation, has failed to show any effect (42). This failure might suggest that the observed autoimmunity in ALS does not contribute to the ongoing degeneration, and that the autoimmunity associated with the disease might in part reflect a failure to recruit an appropriate protective immune mechanism to cope with the threat to the tissue. Accordingly, by boosting a well-controlled immunity that simulates or cross-reacts with weak self-reacting T cells, it is possible to counteract the destructive effect of self-compounds such as glutamate (15, 19, 43). Recent evidence suggests that elements of neurodegenerative and autoimmune disorders can be intermingled in the same disease. Processes known to occur in degenerative diseases have been detected in autoimmune diseases (20, 44–48). It is thus possible that in “mixed” disorders the degenerative tissue will benefit from immunomodulation rather than from immunosuppression (43). Thus, unlike repeated injections as in the therapeutic protocol for MS, a one-time vaccination with Cop-1 can be viewed as a therapy in which immune activity is stimulated and modulated rather than suppressed.

The antibiotic minocycline was recently shown to delay the onset and slow the progression of symptoms in a mouse model of ALS (49–51). In view of the fact that this drug, like other tetracyclines, has various anti-inflammatory actions, its observed beneficial effect in ALS mice might appear to contradict the finding of the present study. It appears, however, that minocycline in the ALS model works by blocking the release of cytochrome c in the mitochondria (50). Thus, the two treatment modalities are not contradictory, and might even be complementary, a possibility that is worth investigating.

The pathogenesis of ALS is thought to be related to an insufficiency of glutamate transporters, whose function is to buffer an excess of extracellular glutamate (52, 53). IFN- γ up-regulates the expression of glutamate transporters by astrocytes (54). Because IFN- γ is a dominant cytokine in T helper 1 cells, our group has

suggested that the activity of T cell-derived IFN- γ might underlie a mechanism whereby the T cells, once they home to the lesion site, are activated, and assist the resident microglia in their task of clearing away cell debris and other toxic substances that threaten the tissue. Studies by our group have indeed demonstrated that the phagocytic activity of microglia and their capacity for uptake of radioactive glutamate are significantly increased after their exposure to IFN- γ (I. Shaked, O. Butovsky, T. Mizrahi, R. Gersner, X. Xiao, P. Soteropoulos, P. Tolias, R. P. Hart, and M.S., unpublished data).

Other studies by our group have shown that Cop-1-reactive T cells, when injected into uninjured rats, home to the CNS, a feature characteristic of T cells that recognize self-antigens (38). The number of Cop-1-reactive T cells that home to the CNS is increased after CNS injury (55). It therefore seems likely that Cop-1-reactive T cells recognize self-antigens presented at the site of the lesion. However, because Cop-1 is not identical in structure to any self-reacting antigen, the T cells it activates are probably those that respond with low affinity, i.e., not the potentially pathogenic ones. It was suggested that Cop-1 acts like an altered peptide ligand in activating nonencephalitogenic T cells (21). The use of Cop-1, which was characterized as a weak self-reacting antigen that cross-reacts with a wide range of self-reacting T cells (20, 21), might be a way to satisfy the diverse requirements among human individuals with respect to safe self-reactive antigens. In a model of glaucoma, a disease characterized by chronic optic nerve neuropathy, Cop-1 vaccination was found to induce a transient increased infiltration of

T cells, and proved itself to be a powerful tool for tissue protection. This was seen when retinas of animals immunized with Cop-1 showed a better morphology than retinas of noninjured animals, both subjected to glutamate toxicity (15). It is therefore conceivable that, as in other CNS insult models, in the present model vaccination transiently increases the number of infiltrating T cells (37), thereby boosting the ability of resident cells to mediate tissue maintenance and repair.

ALS associated with SOD mutations represents only a small fraction of ALS patients. Therefore, the promising results with Cop-1 obtained here in the acute peripheral nerve injury model in addition to its effect in the transgenic mice argue in favor of Cop-1 as a treatment for other forms of ALS besides to the familial disease. On the assumption that the number of causative or risk factors in ALS or any other motor neuron disease is large, it is unlikely that global protection can be achieved by a single drug targeted against a single mediator of toxicity. Global protection, providing the multiple factors needed for CNS recovery, might be obtained by a therapeutic strategy in which the body's protective (immune system) resources are harnessed. Because T cells are prominent among the immune participants in neuroprotection (13, 16), such therapy would presumably recruit a myriad of T cell-derived factors (35, 36).

In view of the present findings, and because Cop-1 has been approved by the Food and Drug Administration for clinical use in MS, we suggest that it should immediately be developed (in a clinically approved formulation and regimen) into a vaccine for the treatment of peripheral nerve injury and motor neuron diseases.

1. Rosen, D. R., Siddique, T., Patterson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J. P., Deng, H. X., et al. (1993) *Nature* **362**, 59–62.
2. Hadano, S., Yanagisawa, Y., Skaug, J., Fichter, K., Nasir, J., Martindale, D., Koop, B. F., Scherer, S. W., Nicholson, D. W., Rouleau, G. A., et al. (2001) *Genomics* **71**, 200–213.
3. Yang, Y., Hentati, A., Deng, H. X., Dabbagh, O., Sasaki, T., Hirano, M., Hung, W. Y., Ouahchi, K., Yan, J., Azim, A. C., et al. (2001) *Nat. Genet.* **29**, 160–165.
4. Ludolph, A. C. & Munch, C. (1999) *Drug Metab. Rev.* **31**, 619–634.
5. Gurney, M. E. (1997) *J. Neurol. Sci.* **152** (Suppl. 1), S67–S73.
6. Subramaniam, J. R., Lyons, W. E., Liu, J., Bartnikas, T. B., Rothstein, J., Price, D. L., Cleveland, D. W., Gitlin, J. D. & Wong, P. C. (2002) *Nat. Neurosci.* **5**, 301–307.
7. Ludolph, A. C., Meyer, T. & Riepe, M. W. (2000) *J. Neurol.* **247**, 17–16.
8. Liu, Z. & Martin, L. J. (2001) *J. Histochem. Cytochem.* **49**, 957–972.
9. Martin, L. J., Price, A. C., Kaiser, A., Shaikh, A. Y. & Liu, Z. (2000) *Int. J. Mol. Med.* **5**, 3–13.
10. Doble, A. & Kennel, P. (2000) *Amyotroph. Lateral Scler. Other Motor Neuron Disord.* **1**, 301–312.
11. Meiningen, V., Lacombiez, L. & Salachas, F. (2000) *J. Neurol.* **247**, 19–22.
12. Hauben, E., Butovsky, O., Nevo, U., Yoles, E., Moalem, G., Agronov, E., Mor, F., Leibowitz-Amit, R., Pevsner, E., Akselrod, S., et al. (2000) *J. Neurosci.* **20**, 6421–6430.
13. Kipnis, J., Yoles, E., Schori, H., Hauben, E., Shaked, I. & Schwartz, M. (2001) *J. Neurosci.* **21**, 4564–4571.
14. Moalem, G., Lebowitz-Amit, R., Yoles, E., Mor, F., Cohen, I. R. & Schwartz, M. (1999) *Nat. Med.* **5**, 49–55.
15. Schori, H., Kipnis, J., Yoles, E., WoldeMussie, E., Ruiz, G., Wheeler, L. A. & Schwartz, M. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 3398–3403.
16. Yoles, E., Hauben, E., Palgi, O., Agronov, E., Gothilf, A., Cohen, A., Kuchroo, V., Cohen, I. R., Weiner, H. & Schwartz, M. (2001) *J. Neurosci.* **21**, 3740–3748.
17. Hauben, E., Agronov, E., Gothilf, A., Nevo, U., Cohen, A., Smirnov, I., Steinman, L. & Schwartz, M. (2001) *J. Clin. Invest.* **108**, 591–599.
18. Hauben, E., Mizrahi, T., Agronov, E. & Schwartz, M. (2002) *Eur. J. Neurosci.* **16**, 1731–1740.
19. Schori, H., Yoles, E., Wheeler, L. A. & Schwartz, M. (2002) *Eur. J. Neurosci.* **16**, 557–564.
20. Kipnis, J. & Schwartz, M. (2002) *Trends Mol. Med.* **8**, 319–323.
21. Hafler, D. A. (2002) *J. Clin. Invest.* **109**, 581–584.
22. Bakala, S., Kipnis, J., Yoles, E. & Schwartz, M. (2002) *Invest. Ophthalmol. Vis. Sci.* **43**, 2648–2653.
23. Dohm, S., Streppel, M., Guntinas-Lichius, O., Pesheva, P., Probstmeier, R., Walther, M., Neiss, W. F., Stennert, E. & Angelov, D. N. (2000) *Restor. Neurol. Neurosci.* **16**, 117–126.
24. Gundersen, H. J. (1986) *J. Microsc.* **143**, 3–45.
25. Sendtner, M., Gotz, R., Holtmann, B., Escary, J. L., Masu, Y., Carroll, P., Wolf, E., Brem, G., Brulet, P. & Thoenen, H. (1996) *Curr. Biol.* **6**, 686–694.
26. Semba, K., Szechtman, H. & Komisaruk, B. R. (1980) *Brain Res.* **195**, 281–298.
27. Welker, W. I. (1964) *Behaviour* **22**, 223–244.
28. Carvell, G. E. & Simons, D. J. (1990) *J. Neurosci.* **10**, 2638–2648.
29. Komisaruk, B. R. (1970) *J. Comp. Physiol. Psychol.* **70**, 482–492.
30. Dorfl, J. (1985) *J. Anat.* **142**, 173–184.
31. Kipnis, J., Mizrahi, T., Hauben, E., Shaked, I., Shevach, E. & Schwartz, M. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 15620–15625.
32. Mizrahi, T., Hauben, E. & Schwartz, M. (2002) *J. Immunology* **169**, 5971–5977.
33. Fisher, J., Levkovich-Verbin, H., Schori, H., Yoles, E., Butovsky, O., Kaye, J. F., Ben-Nun, A. & Schwartz, M. (2001) *J. Neurosci.* **21**, 136–142.
34. Kipnis, J., Mizrahi, T., Yoles, E., Ben-Nun, A. & Schwartz, M. (2002) *J. Neuroimmunol.* **130**, 78–85.
35. Barouch, R. & Schwartz, M. (2002) *FASEB J.* **16**, 1304–1306.
36. Moalem, G., Gdalyahu, A., Shani, Y., Otten, U., Lazarovici, P., Cohen, I. R. & Schwartz, M. (2000) *J. Autoimmun.* **15**, 331–345.
37. Butovsky, O., Hauben, E. & Schwartz, M. (2001) *FASEB J.* **15**, 1065–1067.
38. Kipnis, J., Yoles, E., Porat, Z., Cohen, A., Mor, F., Sela, M., Cohen, I. R. & Schwartz, M. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 7446–7451.
39. Niebroj-Dobosz, I., Domitrz, I. & Mickiewicz, A. (1999) *Folia Neuropathol.* **37**, 107–132.
40. Appel, S. H. & Simpson, E. P. (2001) *Curr. Neurotol. Neurosci. Rep.* **1**, 303–305.
41. Alacidini, A. & Latov, N. (2000) *J. Immunoassay* **21**, 377–386.
42. Drachman, D. B., Fishman, P. S., Rothstein, J. D., Motomura, M., Lang, B., Vincent, A. & McEllis, E. D. (1995) *Adv. Neurol.* **68**, 59–65.
43. Schwartz, M. & Kipnis, J. (2002) *Trends Immunol.* **23**, 530–534.
44. Bjartmar, C. & Trapp, B. D. (2001) *Curr. Opin. Neurol.* **14**, 271–278.
45. Bjartmar, C., Kinkel, R. P., Kidd, G., Rudick, R. A. & Trapp, B. D. (2001) *Neurology* **57**, 1248–1252.
46. De Stefano, N., Narayanan, S., Francis, G. S., Arnaoutelis, R., Tartaglia, M. C., Antel, J. P., Matthews, P. M. & Arnold, D. L. (2001) *Arch. Neurol.* **58**, 65–70.
47. Hohlfeld, R., Kerschensteiner, M., Stadelmann, C., Lassmann, H. & Wekerle, H. (2000) *J. Neuroimmunol.* **107**, 161–166.
48. Steinman, L. (2001) *Nat. Immunol.* **2**, 762–764.
49. Kriz, J., Nguyen, M. D. & Julien, J. P. (2002) *Neurobiol. Dis.* **10**, 268–278.
50. Van Den Bosch, L., Tilkin, P., Lemmens, G. & Robberecht, W. (2002) *NeuroReport* **13**, 1067–1070.
51. Zhu, S., Stavroskaya, I. G., Drozda, M., Kim, B. Y., Ona, V., Li, M., Sarang, S., Liu, A. S., Hartley, D. M., Wu, du, C., et al. (2002) *Nature* **417**, 74–78.
52. Fukamachi, S., Furuta, A., T., I., Ikenoue, T., Kaneko, T., Rothstein, J. D. & Iwaki, T. (2001) *Brain Res. Dev. Brain Res.* **132**, 131–139.
53. Howland, D. S., Liu, J., She, Y., Goad, B., Maragakis, N. J., Kim, B., Erickson, J., Kulik, J., DeVito, L., Psaltis, G., et al. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 1604–1609.
54. Hu, S., Sheng, W. S., Ehrlich, L. C., Peterson, P. K. & Chao, C. C. (2000) *Neuroimmunomodulation* **7**, 153–159.
55. Moalem, G., Monsonego, A., Shani, Y., Cohen, I. R. & Schwartz, M. (1999) *FASEB J.* **13**, 1207–1217.

Therapeutic Vaccination for Closed Head Injury

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ABSTRACT

Closed head injury often has a devastating outcome, partly because the insult, like other injuries to the central nervous system (CNS), triggers self-destructive processes. During studies of the response to other CNS insults, it was unexpectedly discovered that the immune system, if well controlled, provides protection against self-destructive activities. Here we show that in mice with closed head injury, the immune system plays a key role in the spontaneous recovery. Strain-related differences were observed in the ability to harness a T cell-dependent protective mechanism against the effects of the injury. We further show that the trauma-induced deficit could be reduced, both functionally and anatomically, by post-traumatic vaccination with Cop-1, a synthetic copolymer used to treat patients with multiple sclerosis and found (using a different treatment protocol) to effectively counteract the loss of neurons caused by axonal injury or glutamate-induced toxicity. We suggest that a compound such as Cop-1 can be safely developed as a therapeutic vaccine to boost the body's immune repair mechanisms, thereby providing multifactorial protection against the consequences of brain trauma.

Key words: brain injury; autoimmune neuroprotection; strain differences; CNS inflammation; EAE-susceptibility; Cop-1 (Glatiramer acetate)

INTRODUCTION

INJURIES TO THE CENTRAL NERVOUS SYSTEM (CNS) may be grouped according to whether the damage predominantly affects the white matter (axons) or the gray matter (cell bodies or soma) (Schwartz et al., 1999). The outcome of a CNS insult depends not only on the extent of the primary injury but also on the amount of secondary degeneration that subsequently develops (Harrop et al., 2001; Yoles and Schwartz, 1998). Neurons that are directly damaged will inevitably die, regardless of the lo-

cation (white or gray matter) of the lesion. The relatively rapid process of primary death is followed by the secondary degeneration of adjacent neurons that escaped the initial insult. The primary death of neurons and of supportive cells causes an increase in the concentrations of potentially toxic physiological substances such as glutamate and reactive oxygen and nitrogen species, creating a hostile environment for the neighboring neurons (Beattie et al., 2000; Schwartz and Yoles, 2000; Shohami et al., 1997, 1999; Tymianski and Tator, 1996). In addition, at the site of injury alterations of blood flow and im-

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pairment of energy metabolism and ionic homeostasis occur (Assaf et al., 1999; Mautes et al., 2001; Preston et al., 2001). These factors, among others, mediate the spread of secondary neurodegeneration. Treatments that prevent this propagation of damage are described as neuroprotective.

The CNS of mammals is characterized by "immune privilege," a unique evolutionary status that restricts immune cell invasion of the intact CNS (Neumann, 2000; Streilein, 1995; Streilein et al., 2000). The finding that memory T cells patrol the healthy CNS (though they do not normally accumulate there) indicates that the barrier to immune cells is not absolute (Neumann et al., 1998; Wekerle et al., 1996). After injury, however, the disrupted blood-brain barrier (BBB) (Chen et al., 1996) freely allows the entry of lymphocytes and other immune cells, which then accumulate in the damaged tissue (Moalem et al., 1999a; Stahel et al., 2000). Inflammation of uninjured CNS tissue is seen in patients with autoimmune diseases such as multiple sclerosis (MS) and in rodents with experimental autoimmune encephalomyelitis (EAE), an animal model for MS (Bar-Or et al., 1999; O'Connor et al., 2001). Until quite recently, it was generally believed that immune invasion of the damaged CNS adversely affects recovery by promoting secondary degeneration (Dal Canto et al., 2000; Onuki et al., 2001; Popovich et al., 1997, 1999). Immune suppression was accordingly thought to be neuroprotective. However, immunosuppressive treatment was found to be effective, if at all, only when applied immediately after the trauma, becoming progressively less effective with time (Benton et al., 2001; Oudega et al., 1999; Solberg et al., 1999; Yoon et al., 1999).

An increasing body of recent evidence suggests that under certain conditions, immune interaction with the injured CNS has a neuroprotective effect (Hammarberg et al., 2000; Schwartz and Moalem, 2001; Schwartz, 2001a,b). In rats subjected to partial crush of the optic nerve or contusive injury of the spinal cord, passive transfer of T cells specific to CNS myelin-associated self-antigens ("autoimmune" T cells) results in long-lasting protection from secondary degeneration (Butovsky et al., 2001; Hauben et al., 2000; Kipnis et al., 2000; Moalem et al., 1999b). This protection occurs even though transfer of these T cells may induce transient EAE (Antel and Owens, 1999; Ben-Nun et al., 1996). It was shown, however, that the autoimmune T cells inducing the neuroprotective response need not be pathogenic, as the response can be safely induced by T cells reactive to a harmless modified peptide of myelin proteins (Fisher et al., 2001; Hauben et al., 2001; Kipnis et al., 2000; Moalem et al., 1999b; Schori et al., 2001a). Further studies have shown that neuroprotection after optic nerve crush injury or after direct exposure of retinal ganglion

cells (RGCs) to glutamate-mediated toxicity can also be conferred by vaccination with copolymer 1 (Cop-1), a compound that cross-reacts with myelin basic protein (MBP) (Kipnis et al., 2000; Schori et al., 2001a).

More recent studies have shown that immune neuroprotection is not merely the result of an experimental manipulation but is the body's physiological response to trauma in experimental models of white or gray matter injuries (Schwartz and Kipnis, 2001; Schwartz and Moalem, 2001; Yoles et al., 2001). Autoimmune T cells are evoked spontaneously by CNS trauma and are controlled by naturally occurring regulatory CD4⁺CD25⁺T cells, which prevent the development of autoimmune diseases (Kipnis et al., 2002a; Schwartz and Kipnis, 2002). A possible explanation for the puzzling dichotomous effect of autoimmune T cells was provided by studies showing that the ability to manifest a spontaneous autoimmune response to CNS trauma is closely correlated with the individual's ability to resist autoimmune disease development (Kipnis et al., 2001; Lundberg et al., 2001; Schwartz and Kipnis, 2001). Rats or mice that are susceptible to the development of EAE lack an efficient mechanism for controlling the spontaneous ability to evoke an injury-induced autoimmune response. Consequently, their T cell-mediated autoimmune response to injury is ineffective in terms of neuronal protection but is amenable to boosting by passive or active immunization.

Cop-1 is a synthetic amino acid polymer (4.7–11 kDa) composed of the amino acids L-alanine, L-lysine, L-glutamic acid, and L-tyrosine, in a molar ratio of 4.2:3.4:1.4:1.0 (Weiner, 1999). It was originally designed to induce EAE, thereby simulating MBP, but was found to be non-encephalitogenic and even to suppress MBP-induced EAE (Weiner, 1999). It also proved useful in the treatment of multiple sclerosis (Farina et al., 2001; Neuhaus et al., 2000). Cop-1 blocks chronic relapsing EAE induced in an (SJL/J × Balb/c/OLA) F₁ mouse model by application of mouse spinal cord homogenate or encephalitogenic peptides of proteolipid protein (Aharoni et al., 1998). It binds with the relevant major histocompatibility complex proteins and leads to the activation of T suppressor cells, which are triggered by determinants common to Cop-1 and MBP (Aharoni et al., 1997, 1999; Sela, 1999; Teitelbaum et al., 1999).

In the present study, we examined the effect of immune system activation on the outcome of closed head injury (CHI). We found strain-related differences in the spontaneous ability of mice to fight off the consequences of CHI. We further found, by actively immunizing mice with Cop-1, that the immune response evoked by Cop-1 is neuroprotective, effectively reducing the spread of damage caused by the brain trauma.

MATERIALS AND METHODS

Animals

Inbred male adult C57Bl/6J and Balb/c/OLA mice (8–12 weeks old) were supplied by the Animal Breeding Center of the Weizmann Institute of Science. Mice were housed in a light- and temperature-controlled room and handled according to the regulations formulated by IACUC (Institutional Animal Care and Use Committee). Animals were matched for age in each experiment.

Antigens

Cop-1 (Copaxone[®]) was purchased from Teva Pharmaceuticals (Petah Tikva, Israel).

Immunization

Mice were injected with 100 µg of Cop-1 emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 5 mg/mL of *Mycobacterium tuberculosis* H37 RA (Difco). Control mice were injected with phosphate-buffered saline (PBS) emulsified in CFA. The emulsion (total volume 0.2 mL) was injected intramuscularly into the flank.

Brain Injury

Experimental CHI was inflicted using a weight-drop device (Chen et al., 1996). Mice were anesthetized with ether, and anesthesia was confirmed by loss of pupillary and corneal reflexes. Following a midline longitudinal incision, the skin was retracted and the skull exposed. The left anterior frontal area was identified and a tipped Teflon cone was placed approximately 1 mm lateral to the midline in the mid-coronal plane. The head was fixed, and a 75-g weight was dropped on the cone from a height of 18 cm, resulting in a relatively severe focal injury to the left hemisphere, corresponding to a value of 4–6 on the human scale (range, 3–15) of the Glasgow Coma Score (Beni-Adani et al., 2001). The mice received supportive oxygenation with 100% O₂ for no longer than 2 min and were then returned to their cages.

Neurological Severity Score

The clinical status of the injured mice was evaluated according to a set of criteria for testing reflexes and motor functions. According to this method, motor ability, balancing, and alertness are evaluated by the mouse's performance of ten motor and behavioral tasks, yielding a neurological severity score (NSS). One point is awarded for failing to perform a particular task (Table 1). Sham-operated mice typically score zero, as they are able to perform all the tasks. The severity of the injury is re-

flected by the NSS at 1 h; values obtained thereafter are usually lower, as the clinical status spontaneously improves during the recovery period. We evaluated the NSS at 24 h, 48 h, and 7 days after the injury in one experiment, and at 14, 19, and 27 days in another. The difference (defined as ΔNSS) between the NSS values at 1 h and at any time point thereafter reflects the extent and speed of recovery of the tested mice. Thus, higher ΔNSS values denote better recovery. This is a useful tool for the evaluation of drug efficacy or genetic manipulation (Beni-Adani et al., 2000; Panikashvili et al., 2001; Tehranian et al., 2002).

Magnetic Resonance Imaging

The MRI procedure was based on a protocol used previously (Assaf et al., 1999) for the same model. Six weeks after the injury, mice ($n = 5$) were anesthetized and scanned *in vivo* in a Bruker 4.7T Biospec scanner. T2-weighted and diffusion-weighted images were obtained from nine axial slices, each 1 mm thick, covering the entire brain. The acquisition parameters are as recorded in Figure 1.

MRI Analysis

The MRI analysis was designed to quantify the volume of damage by automated recognition of affected areas (Fig. 1a). In defining a damaged area in an image, some technical problems are encountered, such as different amplifications for different slices, non-uniform intensities within a slice, and background noise. To overcome these problems we applied the following image normalization pre-processing procedure for the T2-

**TABLE 1. NEUROLOGICAL SEVERITY SCORE (NSS)
FOR MICE WITH CLOSED HEAD INJURY**

Symptom	NSS (points)
Presence of mono- or hemiparesis	1
Inability to walk on a beam 3 cm wide	1
Inability to walk on a beam 2 cm wide	1
Inability to walk on a beam 1 cm wide	1
Inability to balance on a beam 0.5 cm wide	1
Inability to balance on a round stick 0.5 cm in diameter	1
Failure to exit a 50-cm-diameter circle within 2 min	1
Inability to walk straight	1
Loss of startle reflex	1
Loss of seeking behavior	1
Maximum total	10

For each failed task, the mouse receives 1 point. A score of 10 reflects failure in all tasks, and 0 reflects success in all tasks.

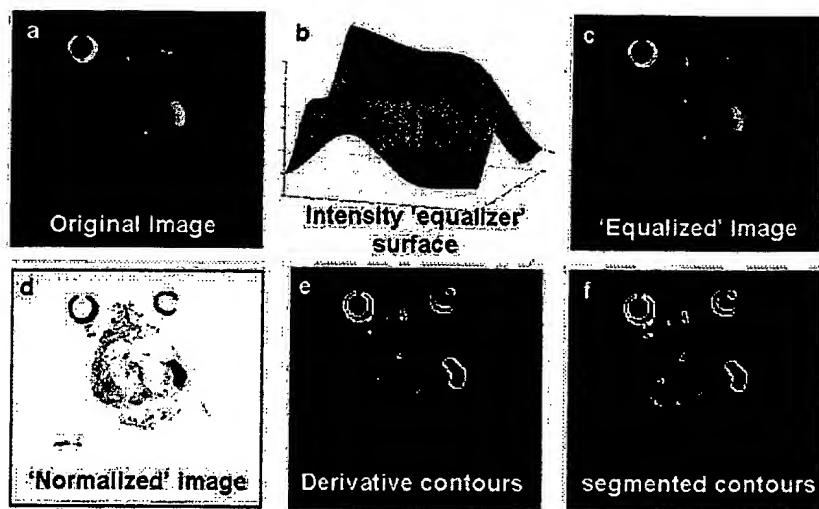


FIG. 1. Magnetic resonance imaging analysis. (a) Original image: spin echo, T2-weighted image with TR/TE = 4000/80, two averages, FOV 2.5×2.5 cm, and matrix size 128×128 pixels. (b) Block-averaged two-dimensional surface for background intensity equalization, averaged over 16×16 pixels and interpolated. (c) Background equalized image: the image was equalized by subtraction of the background surface from the original image. (d) Normalization of the intensities within each slice. (e) Boundaries of "patches," obtained by applying the derivative operator over the image. (f) Map of segmented contours, defined by segmenting the detected contour points using a threshold that was defined by the c-means algorithm.

weighted images (the entire process was performed without utilizing user-defined thresholds or expert-defined regions). Using the c-means algorithm we clustered the intensities within each slice, thereby defining a threshold for elimination of low-intensity (i.e., low SNR) pixels. By block-processing each slice, we obtained a two-dimensional surface describing the global non-uniformities in the intensities (Fig. 1b). This curved surface was bicubically interpolated and subtracted from the image, to reduce the non-uniform component of the image (Fig. 1c). To allow application of a common threshold at a later stage, the intensities within each slice were fitted to the normal distribution, and transformed to normalized probabilities (Fig. 1d). A morphological low-pass filter was used to smooth data with minimal smoothing of the edges. Following this pre-processing, the images were further processed to locate "patches" that correspond to hyperintense areas of damaged tissue. The boundaries of patches were defined by the use of a derivative operator (Fig. 1e). The resulting boundaries were segmented, using a c-means-defined threshold. Isolated points and free-end lines were removed and the resulting patches were labeled (Fig. 1f). Patch areas were calculated and summed to obtain an estimated volume. Inter-slice accumulation was calculated for interconnected patches only, starting from a single reference point manually defined in a damaged site. Areas of damage in the T2-weighted images were qualitatively compared to the areas in the diffusion-weighted images.

The analysis outlined above has a few drawbacks. It is insensitive to tissue elimination due to necrosis, and the calculated volume takes into account only those damaged areas that are in contact with the site of primary injury.

Statistical Analysis

Δ NSS values are expressed as means \pm SEM and were analyzed using the non-parametric Mann-Whitney *U* test.

RESULTS

Spontaneous Neurological Recovery after Closed Head Injury Varies among Strains and Is Correlated with Resistance to Autoimmune Diseases in the CNS

Mice of two strains ($n = 20$ per group) differing in their ability to withstand the consequences of axonal injury or glutamate toxicity (Kipnis et al., 2001; Schori et al., 2001b) were subjected to a well-controlled head injury. Uniformity of the insult was verified by the similarity of NSS values obtained 1 h after the insult. Thereafter, follow-up of the spontaneous recovery, measured by the improvement in neurological status (expressed as Δ NSS), revealed that the Balb/c/OLA (resistant) mice recovered better than the C57Bl/6J (susceptible) mice ($p < 0.05$; Fig. 2). This finding is in line with the previously observed recovery of rodents from other CNS insults (Kipnis et al., 2001).

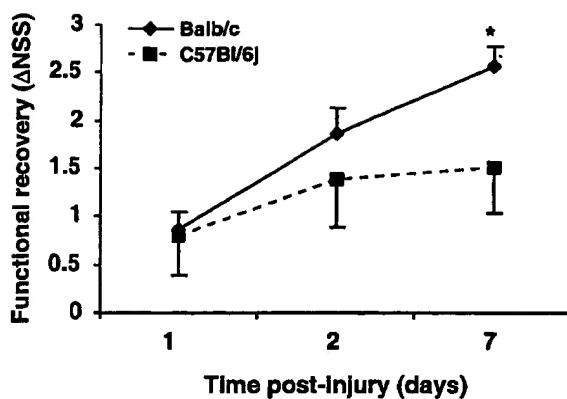


FIG. 2. Better spontaneous recovery after closed head injury in Balb/c mice than in C57Bl/6J mice. Balb/c/OLA and C57Bl/6J mice were subjected to closed head trauma of identical severity. Recovery was assessed on the basis of a neurological severity score. The outcome of brain injury, assessed 7 days after the injury, was significantly better in the EAE-resistant mice than in the C57Bl/6J mice ($p < 0.05$; Mann-Whitney test). The result is a combination of two independent experiments ($n = 8-10$ for each strain in each experiment).

Vaccination with Cop-1 Improves the Functional Outcome of CHI

Our previous findings in models of white matter (axonal) insult and of toxic glutamate insult to gray matter (myelin-free neurons) demonstrated that an immune-mediated protective response can be safely boosted by the use of Cop-1. Cop-1 vaccination apparently circumvents the tissue-specificity barrier to therapeutic vaccines after a CNS insult (Schori et al., 2001b). It was therefore of interest to determine whether this copolymer is therapeutic in cases of head trauma as well (Kipnis et al., 2000; Schori et al., 2001a). Mice of each strain were divided into three groups (Fig. 3) and then subjected to CHI. In both strains, mice that were vaccinated, 7 days prior to injury, with Cop-1 emulsified in CFA showed better functional outcome than control mice that were untreated or were injected with PBS emulsified in CFA. The two strains differed significantly, however, in the rate of recovery. In Balb/c/OLA mice, Δ NSS was significantly greater in the Cop-1-treated than in PBS-treated mice at 2 and 7 days after injury (Fig. 3A; $p < 0.05$ and 0.01 , respectively; Mann-Whitney U test). In contrast, the recovery of C57Bl/6J mice, whether vaccinated with Cop-1 or treated with PBS, was much slower (Fig. 3B). Immunization of naïve mice with Cop-1 emulsified in CFA did not affect their behavioral scores (data not shown).

Since pre-immunization is not a relevant therapeutic approach in cases of brain injury it was of interest to determine whether immunization performed after the insult

was also effective. Immediately after CHI, Balb/c/OLA mice were immunized with Cop-1 in CFA, or injected with PBS in CFA, or not treated, and their clinical recovery was followed for 7 days (Fig. 4A). One week after injury and treatment, the difference in brain injury out-

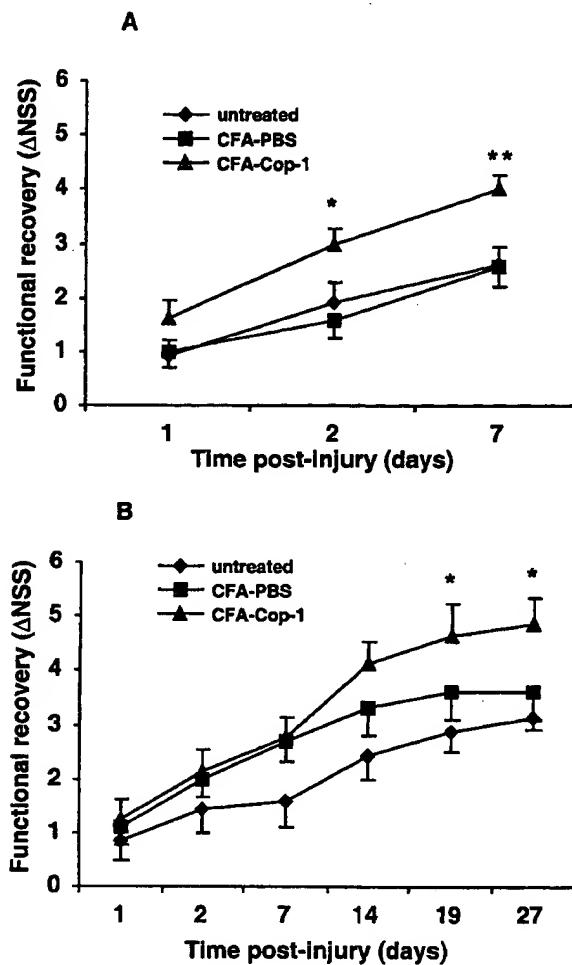


FIG. 3. Vaccination with Cop-1 confers neuroprotection on the damaged brain tissue. Mice were immunized in the flank with $100 \mu\text{g}$ of Cop-1 emulsified in CFA 10 days before brain injury was inflicted. Starting 24 h after the injury, neurological behavior was assessed in three groups of brain-injured mice [treated with Cop-1 emulsified in CFA, injected with PBS in CFA ("sham-immunized"), and untreated]. Two days after brain injury, Balb/c/OLA mice immunized with Cop-1 (A) showed significantly better recovery ($p < 0.05$; Mann-Whitney test) than sham-immunized or untreated mice. By the 7th day after injury, the difference between the sham-immunized and the treated groups was even more significant ($p < 0.01$; Mann-Whitney test). In brain-injured C57Bl/6J mice, the outcome in the group immunized with Cop-1 was not better than that in the sham-immunized or untreated groups for the first 2 weeks after injury (B). Differences between the Cop-1-immunized group and the sham-immunized or untreated groups became significant ($p < 0.05$; Mann-Whitney test) only 19 days after the injury was inflicted, and were sustained until day 27.

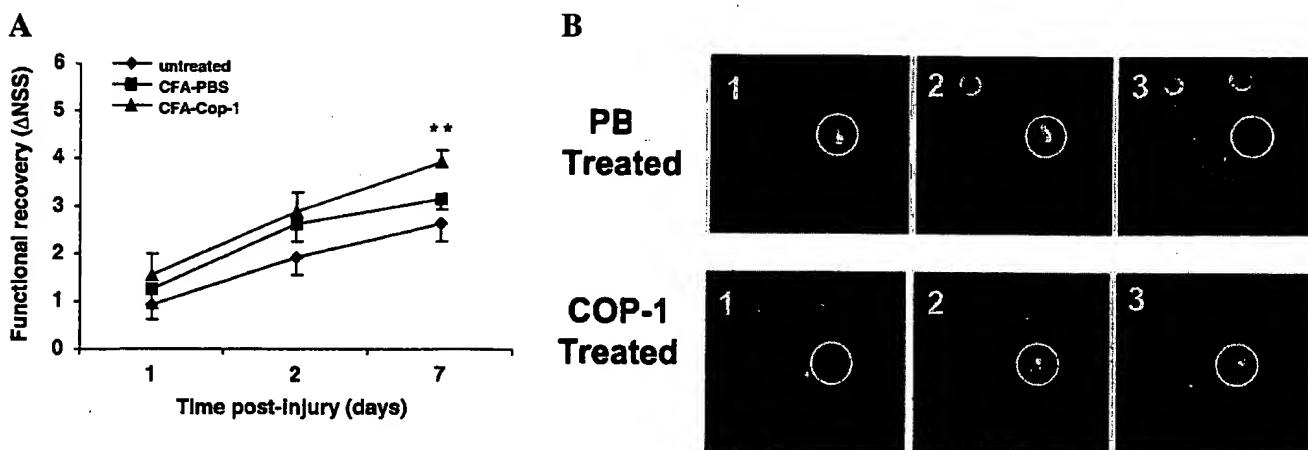


FIG. 4. Vaccination with Cop-1 immediately after brain injury prevents the spread of damage and preserves the neuronal tissue as indicated by MRI. Immediately after brain injury, Balb/c/OLA were immunized in the flank with 100 µg of Cop-1 emulsified in CFA. Seven days later, the immunized group showed significantly better recovery (based on neurological severity scores) than sham-immunized or untreated mice ($p < 0.01$; Mann-Whitney U test) (A). Six weeks after injury, mice were scanned by T2-weighted MRI. Micrographs show images of three different layers taken from one of six Cop-1-treated mice or from one of seven PBS-treated mice. From each scan, the three slices with the largest damaged areas are presented. The area of damage was larger in the PBS-injected mice than in the Cop-1-immunized mice (B).

come between the groups became significant (Fig. 4B; $p < 0.01$). To verify the clinical findings, three mice chosen randomly from each group were evaluated, 6 weeks after CHI, by magnetic resonance imaging (MRI). The results showed that the damaged areas in the Cop-1-vaccinated mice were significantly smaller ($2.1 \pm 0.75 \text{ mm}^3$; mean \pm SD) than in the PBS-treated mice ($5.1 \pm 1.43 \text{ mm}^3$; Fig. 4B). The areas of damage demonstrated in diffusion-weighted images overlapped those seen in T2-weighted images (Fig. 5). As expected, in the T2-weighted images the overall intensity of the injured left hemisphere was greater than that in the right hemisphere, despite manipulation of the images to minimize intensity non-uniformities. In agreement with other studies using the same CHI model (Beni-Adani et al., 2001), we obtained hyperintense damaged areas in the non-injured right hemisphere, possibly reflecting secondary degeneration.

Using MRI techniques and H&E staining in the same experimental model, we previously demonstrated that the best correlation with diffusion-weighted images at all time points was obtained with CHI-induced histological damage visualized 1 week after the injury. This was true for both rats (Assaf et al., 1999) and mice (Beni-Adani et al., 2001).

DISCUSSION

The results of this study showed differences between two mouse strains in their ability to withstand the consequences of closed head trauma. On the basis of our pre-

vious experience with optic nerve injury and glutamate toxicity, we attribute this difference to differences in the ability of the mouse T cells to mediate a protective immune response (Kipnis et al., 2001; Schori et al., 2001b). After CNS injury and challenge with any myelin antigens, a process of T cell-dependent immune neuroprotection appears to be spontaneously elicited in a strain that is genetically resistant to the development of CNS autoimmune diseases, but evoked only to a limited extent in inherently susceptible strains (Kipnis et al., 2001; Schwartz and Kipnis, 2002). The findings of this study thus suggest that the functional outcome of head trauma is similarly affected by genes that are associated with control of the immune response.

Recent findings suggest that the ability to manifest a protective autoimmune response after a CNS insult is controlled by naturally occurring regulatory CD4⁺CD25⁺ T cells (Kipnis et al., 2002a; Schwartz and Kipnis, 2002). Inactivation of these regulatory T cells or vaccination with a suitable antigen can augment the spontaneous neuroprotective response, which—even in individuals capable of manifesting a beneficial autoimmunity—is relatively weak at best, and needs to be boosted. Immunization with Cop-1 in this study indeed resulted in better recovery from CHI in both strains.

Active immunization with the synthetic copolymer Cop-1 in this study resulted in immune neuroprotection in both of the tested mouse strains. Clinical recovery, as expressed by improvement in NSS, was significantly more rapid and more substantial in mice pre-immunized with Cop-1 than in PBS-treated mice. The treatment was ef-

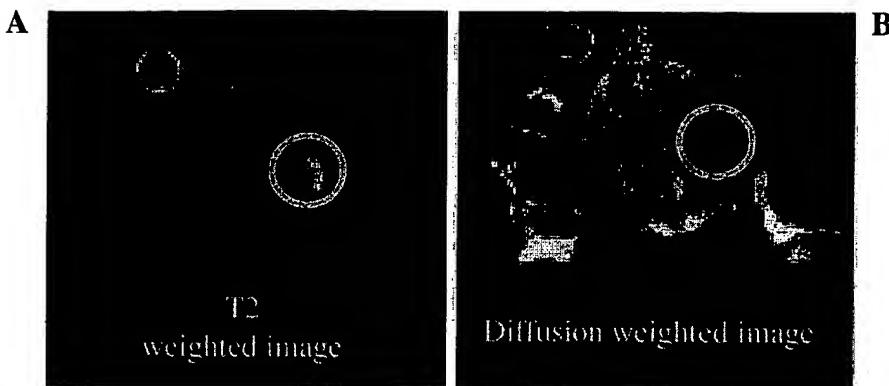


FIG. 5. T2-weighted and diffusion-weighted images. Comparison of a T2-weighted image (A), showing hyperintensity of the damaged area, with a diffusion-weighted image of the same slice (stimulated echo, TR/TE = 3000/40.6, four averages, $G_d = 50$ mT/m). (B). The damaged area in the diffusion-weighted image is defined by hypointensity. The images depict overlapping of the damaged area in both types of acquisitions.

fective even when applied after the injury, suggesting that there is a clinically relevant therapeutic window for immune-mediated neuroprotection after CHI.

Cop-1 vaccination was previously shown to confer neuroprotection in the rat or mouse model of optic nerve crush injury, in rat or mouse RGCs directly exposed (by intraocular injection) to glutamate-mediated toxicity, and after neuronal degeneration in a glaucoma-like rat model of ocular hypertension (Kipnis et al., 2000; Schori et al., 2001a). The results of the present study showed that immunization with Cop-1 is followed by a decrease in the propagation of damage induced by CHI. The mouse strain better able to withstand injurious conditions (Balb/c) responded rapidly to the Cop-1 vaccination, and 24 h after the trauma we could already detect a difference between the Cop-1-vaccinated and the control groups. In C57Bl/6J mice, the strain less resistant to injurious conditions, the response to the treatment was weaker and slower; nevertheless, once a steady state was reached significantly more neuroprotection was evident in the Cop-1-immunized mice than in the PBS-treated controls. By analyzing the MRI images, we were able to delineate the regions of damaged tissue and estimate the volume of the central site of damage. MRI images are closely correlated with histological data from spinal cord injury models (Hauben et al., 2000; Nevo et al., 2001). Even allowing for inaccuracies, the calculated results showed clear differences between Cop-1-treated mice and controls. Inaccuracies in assessment were mainly due to underestimation of the damaged areas.

Studies of T cell-based neuroprotection after optic nerve insult and spinal cord injury have shown that, after an injury, T cells home to the site of damage (Butovsky et al., 2001; Moalem et al., 1999a). These findings led to the suggestion that in order to exert their

neuroprotective effect, T cells have to home to the lesion site and become activated there by their specific antigens, which are presented to them by the relevant antigen-presenting cells. Recent data obtained in our laboratory suggest that this local immune response is Th1-dependent (Kipnis et al., 2002b), and that it serves to activate resident microglia, thereby increasing their capacity for buffering glutamate and for general phagocytosis (Shaked et al., unpublished data). It also increases the production of neurotrophic factors by microglia or other resident cells (Barouch et al., 2002; Moalem et al., 2001). In seeking a way to boost a protective effect of autoimmune T cells, a number of compounds were tested, among them Cop-1. T cells specific to Cop-1 were found to be present in the undamaged CNS, similarly to T cells specific to myelin antigens (though in smaller quantities) (Kipnis et al., 2000; Aharoni et al., 2002). Moreover, after local damage (caused for example by injection of glutamate), treatment with Cop-1 was found to increase the infiltration of immune cells into the lesion site (Schori et al., 2001a). It thus appeared that Cop-1 could safely simulate the effect of myelin antigen in augmenting the relevant local immune response.

From the results of all the studies carried out to date, it appears that the endogenous T cell response evoked by CNS insults has a similar function at the different sites of CNS damage. However, the antigenic specificity of the T cells that are evoked spontaneously may differ from one site to another (Schori et al., 2001a; Mizrahi et al., 2002). In the case of axonal injury, both the endogenous (insult-evoked) T cell response and the exogenous therapeutic T cells are directed to myelin-associated antigens (Kipnis et al., 2000, 2002b). The antigenic specificity of the endogenous T-cell response evoked by CHI has yet to be discovered. It seems, however, that regardless of

whether the insult is inflicted on the gray or the white matter, it can benefit from T cells specific to Cop-1. This compound, being a random copolymer, has a heterogeneous composition. The heterogeneity might explain why Cop-1-reactive T cells can function at different sites, unlike T cells specific to an antigen with a homogeneous molecular structure (Kipnis and Schwartz, 2002). It is also possible that Cop-1-reactive T cells might act as bystander modulators of the endogenous evoked response (Aharoni et al., 1998). Whatever the mechanisms operating in the present study, our results argue in favor of immune involvement in neuroprotection. Like previous results in the optic nerve and the spinal cord (Hauben et al., 2001; Hauben and Schwartz, 2003), the present findings would support the therapeutic use of immunomodulators rather than immunosuppressors, so as to maximize the benefit from the immune system rather than eliminate any possible immune-mediated effect (Schwartz and Kipnis, 2002). It is important to note, however, that the Cop-1 administration protocol used for patients with MS does not lead to neuroprotection. Studies are currently in progress to establish the optimal formulation and protocol required to safely boost the beneficial autoimmune response for neuroprotection.

It was postulated that the T cell-mediated protective response evoked by CNS insults is harnessed to assist local innate immune mechanisms to cope with the insult-induced secretion of self-destructive compounds (Nevo et al., 2003). This T cell-mediated response needs to be rigorously regulated in order to provide protection without risk of autoimmune disease induction. Vaccination with Cop-1 appears to provide a safe way both to regulate and to boost the response. A principal role of the immune system is to efficiently eliminate dead cells and cell debris, and in addition to remove viable cells at the periphery of the injury site, which would otherwise be targeted by the agents of self-destruction and hence become the starting-point of a second wave of self-destructive activity. Protective immunity provides a way to arrest this phase of degeneration, but at some cost.

The process of nerve degeneration is chaotic and involves the activity of numerous physiological compounds. Some of them, though normally essential for brain function (e.g., glutamate), become toxic when (as a result of the insult) their normal concentrations are exceeded. Pharmacological intervention aimed at reducing the toxicity of a particular compound is likely to be accompanied by an undesirable disruption of that compound's normal functioning. Protective autoimmunity appears to be the body's mechanism of coping with conditions of stress, such as those caused by closed head injury. Taken together with earlier findings, the results of this study further support the

contention that the immune response evoked by CNS trauma is always at least potentially beneficial, but it needs to be properly regulated for the beneficial effect to be expressed. If properly regulated and suitably boosted, protective autoimmunity is therefore likely to provide a global therapeutic effect. Since Cop-1 has already been approved for clinical use, it would appear that there are no serious obstacles to its immediate development for the treatment of closed head injury.

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REFERENCES

- AHARONI, R., TEITELBAUM, D., SELA, M., et al. (1997). Copolymer 1 induces T cells of the T helper type 2 that cross-react with myelin basic protein and suppress experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. USA* **94**, 10821–10826.
- AHARONI, R., TEITELBAUM, D., SELA, M., et al. (1998). Bystander suppression of experimental autoimmune encephalomyelitis by T cell lines and clones of the Th2 type induced by copolymer 1. *J. Neuroimmunol.* **91**, 135–146.
- AHARONI, R., TEITELBAUM, D., ARNON, R., et al. (1999). Copolymer 1 acts against the immunodominant epitope 82–100 of myelin basic protein by T cell receptor antagonism in addition to major histocompatibility complex blocking. *Proc. Natl. Acad. Sci. USA* **96**, 634–639.
- AHARONI, R., MESHORER, A., SELA, M., and ARNON, R. (2002). Oral treatment of mice with copolymer 1 (glatiramer acetate) results in the accumulation of specific Th2 cells in the central nervous system. *J. Neuroimmunol.* **126**, 58–68.
- ANTEL, J.P., and OWENS, T. (1999). Immune regulation and CNS autoimmune disease. *J. Neuroimmunol.* **100**, 181–189.
- ASSAF, Y., HOLOKOVSKY, A., BERMAN, E., et al. (1999). Diffusion and perfusion magnetic resonance imaging following closed head injury in rats. *J. Neurotrauma* **16**, 1165–1176.
- BAR-OR, A., OLIVEIRA, E.M., ANDERSON, D.E., et al. (1999). Molecular pathogenesis of multiple sclerosis. *J. Neuroimmunol.* **100**, 252–259.

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BEATTIE, M.S., FAROOQUI, A.A., and BRESNAHAN, J.C. (2000). Review of current evidence for apoptosis after spinal cord injury. *J. Neurotrauma* **17**, 915–925.

BEN-NUN, A., and COHEN, I.R. (1982). Experimental autoimmune encephalomyelitis (EAE) mediated by T cell lines: process of selection of lines and characterization of the cells. *J. Immunol.* **129**, 303–308.

BEN-NUN, A., MENDEL, I., BAKIMER, R., et al. (1996). The autoimmune reactivity to myelin oligodendrocyte glycoprotein (MOG) in multiple sclerosis is potentially pathogenic: effect of copolymer 1 on MOG-induced disease. *J. Neurol.* **243**, S14–S22.

BENI-ADANI, L., GOZES, I., COHEN, Y., et al. (2001). A peptide derived from activity-dependent neuroprotective protein (ADNP) ameliorates injury response in closed head injury in mice. *J. Pharmacol. Exp. Ther.* **296**, 57–63.

BENTON, R.L., ROSS, C.D., MILLER, K.E. (2001). Spinal taurine levels are increased 7 and 30 days following methylprednisolone treatment of spinal cord injury in rats. *Brain Res.* **893**, 292–300.

BUTOVSKY, O., HAUBEN, E., and SCHWARTZ, M. (2001). Morphological aspects of spinal cord autoimmune neuroprotection: colocalization of T cells with B7-2 (CD86) and prevention of cyst formation. *FASEB J.* **15**, 1065–1067.

CHEN, Y., CONSTANTINI, S., TREMBOVLER, V., et al. (1996). An experimental model of closed head injury in mice: pathophysiology, histopathology, and cognitive deficits. *J. Neurotrauma* **13**, 557–568.

DAL CANTO, M.C., CALENOFF, M.A., MILLER, S.D., et al. (2000). Lymphocytes from mice chronically infected with Theiler's murine encephalomyelitis virus produce demyelination of organotypic cultures after stimulation with the major encephalitogenic epitope of myelin proteolipid protein. Epitope spreading in TMEV infection has functional activity. *J. Neuroimmunol.* **104**, 79–84.

FARINA, C., THEN BERGH, F., ALBRECHT, H., et al. (2001). Treatment of multiple sclerosis with Copaxone (COP): Elispot assay detects COP-induced interleukin-4 and interferon-gamma response in blood cells. *Brain* **124**, 705–719.

FISHER, J., LEVKOVITCH-VERBIN, H., SCHORI, H., et al. (2001). Vaccination for neuroprotection in the mouse optic nerve: implications for optic neuropathies. *J. Neurosci.* **21**, 136–142.

HAMMARBERG, H., LIDMAN, O., LUNDBERG, C., et al. (2000). Neuroprotection by encephalomyelitis: rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. *J. Neurosci.* **20**, 5283–5291.

HARROP, J.S., SHARAN, A.D., VACCARO, A.R., et al. (2001). The cause of neurologic deterioration after acute cervical spinal cord injury. *Spine* **26**, 340–346.

HAUBEN, E., BUTOVSKY, O., NEVO, U., et al. (2000). Passive or active immunization with myelin basic protein promotes recovery from spinal cord contusion. *J. Neurosci.* **20**, 6421–6430.

HAUBEN, E., AGRANOV, E., GOTHLIF, A., et al. (2001). Posttraumatic therapeutic vaccination with modified myelin self-antigen prevents complete paralysis while avoiding autoimmune disease. *J. Clin. Invest.* **108**, 591–599.

HAUBEN, E., and SCHWARTZ, M. (2003). Therapeutic vaccination for spinal cord injury: Helping the body to cure itself. *Trends Pharmacol. Sci.* **24**, 7–12.

KIPNIS, J., YOLES, E., PORAT, Z., et al. (2000). T cell immunity to copolymer 1 confers neuroprotection on the damaged optic nerve: possible therapy for optic neuropathies. *Proc. Natl. Acad. Sci. USA* **97**, 7446–7451.

KIPNIS, J., YOLES, E., SCHORI, H., et al. (2001). Neuronal survival after CNS insult is determined by a genetically encoded autoimmune response. *J. Neurosci.* **21**, 4564–4571.

KIPNIS, J., MIZRAHI, T., HAUBEN, E., et al. (2002a). Neuroprotective autoimmunity: naturally occurring CD4⁺ CD25⁺ regulatory T cells suppress the ability to withstand injury to the central nervous system. *Proc. Natl. Acad. Sci. USA* **12**, 12.

KIPNIS, J., YOLES, E., MIZRAHI, T., et al. (2002b). Myelin-specific Th1 cells are necessary for post-traumatic protective autoimmunity. *J. Neuroimmunol.* **130**, 78.

KIPNIS, J., and SCHWARTZ, M. (2002). Dual action of glatiramer acetate (Cop-1) in the treatment of CNS autoimmune and neurodegenerative disorders. *Trends Mol. Med.* **8**, 319–323.

LUNDBERG, C., LIDMAN, O., HOLMDAHL, R., et al. (2001). Neurodegeneration and glial activation patterns after mechanical nerve injury are differentially regulated by non-MHC genes in congenic inbred rat strains. *J. Comp. Neurol.* **431**, 75–87.

MAUTES, A.E., THOME, D., STEUDEL, W.I., et al. (2001). Changes in regional energy metabolism after closed head injury in the rat. *J. Mol. Neurosci.* **16**, 33–39.

MIZRAHI, T., HAUBEN, E., and SCHWARTZ, M. (2002). The tissue-specific self-pathogen is the protective self-antigen: The case of uveitis. *J. Immunol.* **169**, 5971–5977.

MOALEM, G., MONSONEGO, A., SHANI, Y., et al. (1999a). Differential T cell response in central and peripheral nerve injury: connection with immune privilege. *FASEB J.* **13**, 1207–1217.

MOALEM, G., LEIBOWITZ-AMIT, R., YOLES, E., et al. (1999b). Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat. Med.* **5**, 49–55.

MOALEM, G., GDALYAHU, A., SHANI, Y., et al. (2000). Production of neurotrophins by activated T cells: implications for neuroprotective autoimmunity. *J. Autoimmun.* **15**, 331–345.

NEUHAUS, O., FARINA, C., YASSOURIDIS, A., et al. (2000). Multiple sclerosis: comparison of copolymer-1-reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells. *Proc. Natl. Acad. Sci. USA* **97**, 7452–7457.

NEUMANN, H. (2000). The immunological microenvironment in the CNS: implications on neuronal cell death and survival. *J. Neural. Transm. Suppl.* **59**, 59–68.

NEUMANN, H., MISGELD, T., MATSUMURO, K., et al. (1998). Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. *Proc. Natl. Acad. Sci. USA* **95**, 5779–5784.

NEVO, U., KIPNIS, J., GOLDING, I., et al. (2003). Autoimmunity as a special case of immunity: removing threats from within. *Trends Mol. Med.* **9**, 88–93.

O'CONNOR, K.C., BAR-OR, A., HAFLER, D.A. (2001). The neuroimmunology of multiple sclerosis: possible roles of T and B lymphocytes in immunopathogenesis. *J. Clin. Immunol.* **21**, 81–92.

ONUKI, M., AYERS, M.M., BERNARD, C.C., et al. (2001). Axonal degeneration is an early pathological feature in autoimmune-mediated demyelination in mice. *Microsc. Res. Tech.* **52**, 731–739.

OUDEGA, M., VARGAS, C.G., WEBER, A.B., et al. (1999). Long-term effects of methylprednisolone following transection of adult rat spinal cord. *Eur. J. Neurosci.* **11**, 2453–2464.

POPOVICH, P.G., WEI, P., and STOKES, B.T. (1997). Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. *J. Comp. Neurol.* **377**, 443–464.

POPOVICH, P.G., GUAN, Z., WEI, P., et al. (1999). Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. *Exp. Neurol.* **158**, 351–365.

PRESTON, E., WEBSTER, J., and SMALL, D. (2001). Characteristics of sustained blood-brain barrier opening and tissue injury in a model for focal trauma in the rat. *J. Neurotrauma* **18**, 83–92.

SCHORI, H., KIPNIS, J., YOLES, E., et al. (2001a). Vaccination for protection of retinal ganglion cells against death from glutamate cytotoxicity and ocular hypertension: implications for glaucoma. *Proc. Natl. Acad. Sci. USA* **98**, 3398–3403.

SCHORI, H., YOLES, E., SCHWARTZ, M. (2001b). T-cell-based immunity counteracts the potential toxicity of glutamate in the central nervous system. *J. Neuroimmunol.* **119**, 199–204.

SCHWARTZ, M. (2001a). T cell-mediated neuroprotection is a physiological response to central nervous system insults. *J. Mol. Med.* **78**, 594–597.

SCHWARTZ, M. (2001b). Physiological approaches to neuroprotection, boosting of protective autoimmunity. *Surv. Ophthalmol.* **45**, S256–S260.

SCHWARTZ, M., and KIPNIS, J. (2001). Protective autoimmunity: regulation and prospects for vaccination after brain and spinal cord injuries. *Trends Mol. Med.* **7**, 252–258.

SCHWARTZ, M., and KIPNIS, J. (2002). Autoimmunity on alert: naturally occurring regulatory CD4⁺CD25⁺ T cells as a part of the evolutionary compromise between a "need" and a "risk." *Trends Immunol.* **23**, 530–534.

SCHWARTZ, M., MOALEM, G. (2001). Beneficial immune activity after CNS injury: prospects for vaccination. *J. Neuroimmunol.* **113**, 185–192.

SCHWARTZ, M., and YOLES, E. (2000). Self-destructive and self-protective processes in the damaged optic nerve: implications for glaucoma. *Invest. Ophthalmol. Vis. Sci.* **41**, 349–351.

SCHWARTZ, M., YOLES, E., and LEVIN, L. (1999). "Axogenetic" and "somagenic" neurodegenerative diseases: definitions and therapeutic implications. *Mol. Med. Today* **5**, 470–473.

SELA, M. (1999). The concept of specific immune treatment against autoimmune diseases. *Int. Rev. Immunol.* **18**, 201–216.

SHOHAMI, E., BEIT-YANNAI, E., HOROWITZ, M., et al. (1997). Oxidative stress in closed-head injury: brain antioxidant capacity as an indicator of functional outcome. *J. Cereb. Blood Flow Metab.* **17**, 1007–1019.

SHOHAMI, E., GINIS, I., HALLENBECK, J.M. (1999). Dual role of tumor necrosis factor alpha in brain injury. *Cytokine Growth Factor Rev.* **10**, 119–130.

SOLBERG, Y., DUBINSKI, G., TCHIRKOV, M., et al. (1999). Methylprednisolone therapy for retinal laser injury. *Surv. Ophthalmol.* **44**, Suppl 1, S85–S92.

STAHEL, P.F., SHOHAMI, E., YOUNIS, F.M., et al. (2000). Experimental closed head injury: analysis of neurological outcome, blood-brain barrier dysfunction, intracranial neutrophil infiltration, and neuronal cell death in mice deficient in genes for pro-inflammatory cytokines. *J. Cereb. Blood Flow Metab.* **20**, 369–380.

STREILEIN, J.W. (1995). Unraveling immune privilege. *Science* **270**, 1158–1159.

STREILEIN, J.W., OKAMOTO, S., SANO, Y., et al. (2000). Neural control of ocular immune privilege. *Ann. NY Acad. Sci.* **917**, 297–306.

TEHRANIAN, R., ANDELL-JONSSON, S., BENI, S.M., et al. (2002). Improved recovery and delayed cytokine induction after closed head injury in mice with central overexpression of the secreted isoform of the interleukin-1 receptor antagonist. *J. Neurotrauma* **19**, 939–951.

TEITELBAUM, D., ARNON, R., and SELA, M. (1999). Im-

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munomodulation of experimental autoimmune encephalomyelitis by oral administration of copolymer 1. Proc. Natl. Acad. Sci. USA **96**, 3842–3847.

TYMIANSKI, M., and TATOR, C.H. (1996). Normal and abnormal calcium homeostasis in neurons: a basis for the pathophysiology of traumatic and ischemic central nervous system injury. Neurosurgery **38**, 1176–1195.

WEINER, H.L. (1999). Oral tolerance with copolymer 1 for the treatment of multiple sclerosis. Proc. Natl. Acad. Sci. USA **96**, 3333–3335.

WEKERLE, H., BRADL, M., LININGTON, C., et al. (1996). The shaping of the brain-specific T lymphocyte repertoire in the thymus. Immunol. Rev. **149**, 231–243.

YOLES, E., and SCHWARTZ, M. (1998). Degeneration of spared axons following partial white matter lesion: implications for optic nerve neuropathies. Exp. Neurol. **153**, 1–7.

YOLES, E., FRIEDMANN, I., BAROUCH, R., et al. (2001). Self-protective mechanism awakened by glutamate in retinal ganglion cells. J. Neurotrauma **18**, 339–349.

YOON, D.H., KIM, Y.S., and YOUNG, W. (1999). Therapeutic time window for methylprednisolone in spinal cord injured rat. Yonsei Med. J. **40**, 313–320.

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36. Use according to any one of claims 27 to 33, wherein said active agent is poly-Glu,Tyr.
- 5 37. Use according to any one of claims 27 to 36, wherein said vaccine is for administration at least once a month.
38. Use according to any one of claims 27 to 36, wherein said vaccine is for administration at least once every 2-3 months.

10

39. Use according to any one of claims 27 to 38, wherein said vaccine is for administration with another drug for treatment of MND such as Riluzole.

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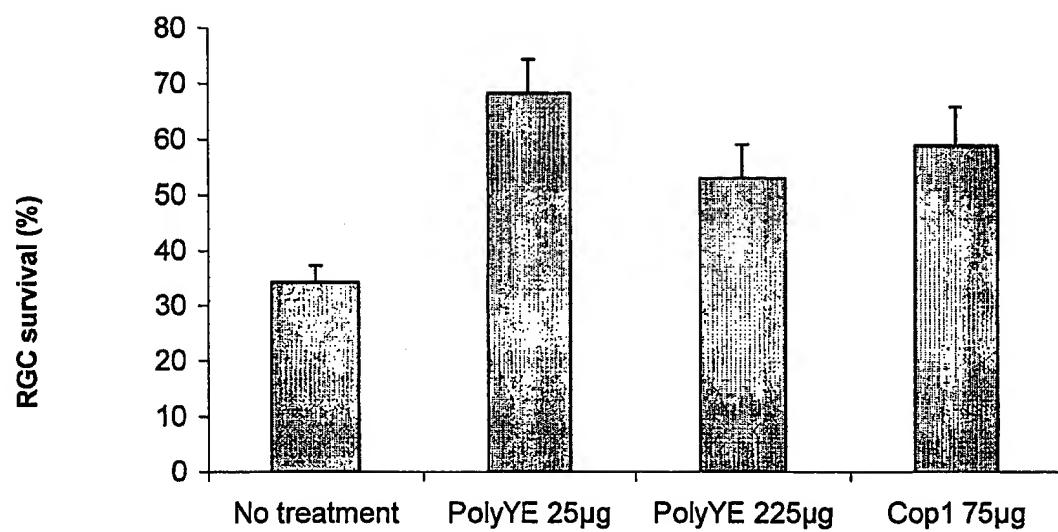


Fig. 1

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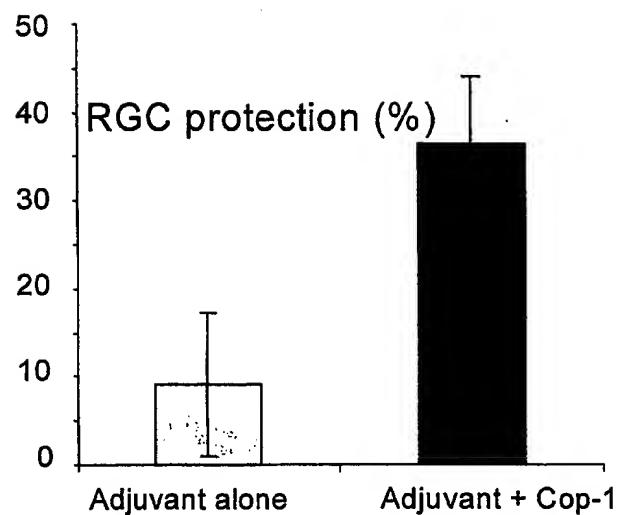


Fig. 2A

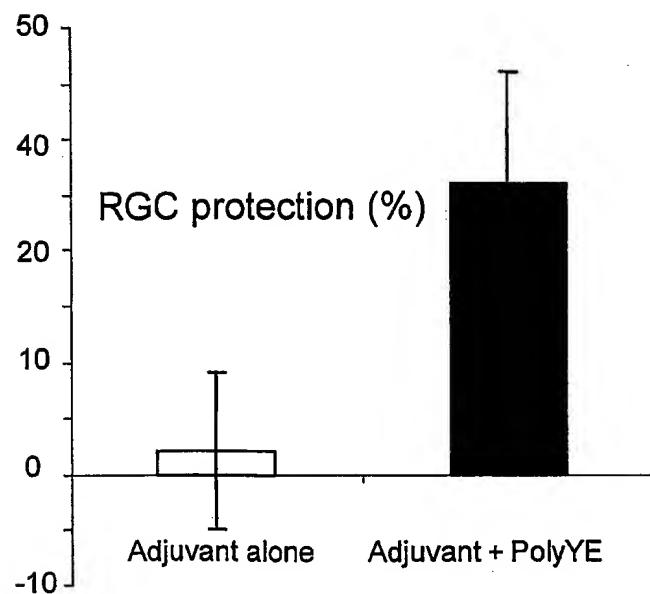


Fig. 2B

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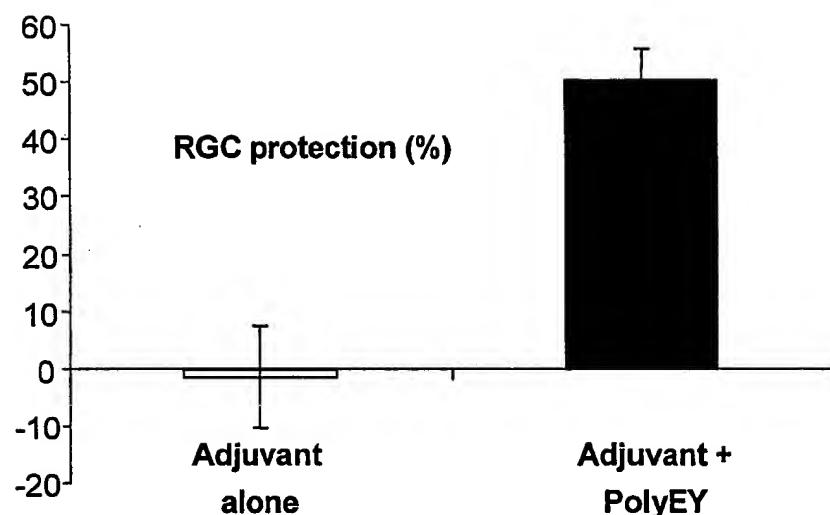


Fig. 3A

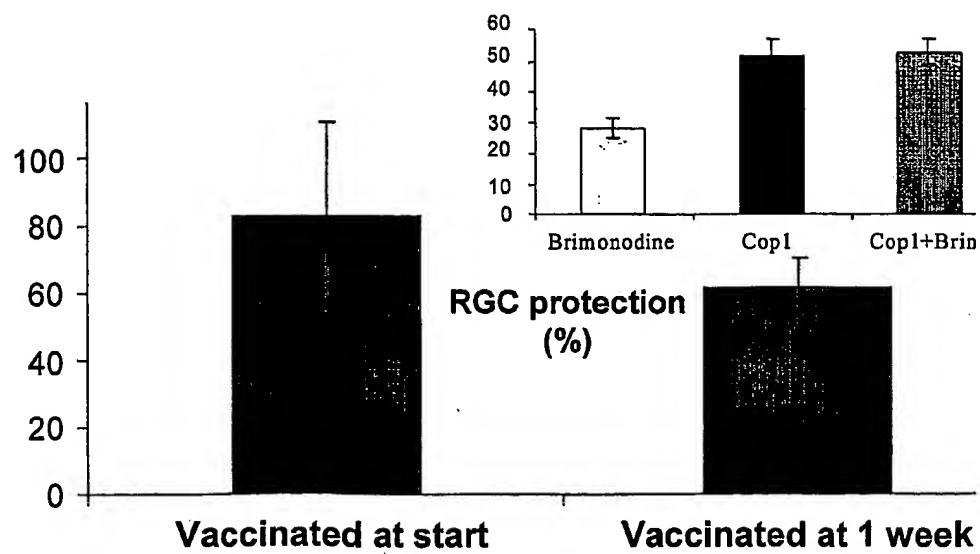


Fig. 3B

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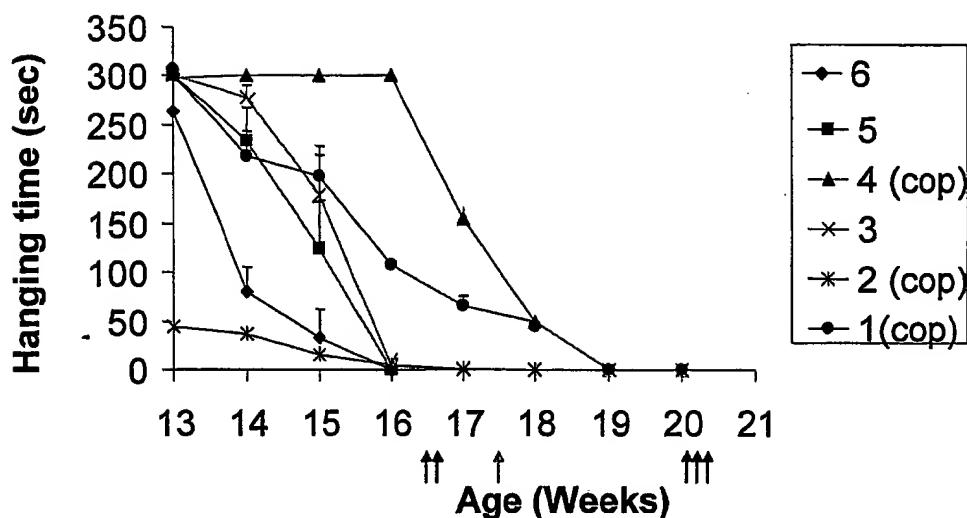


Fig. 4A

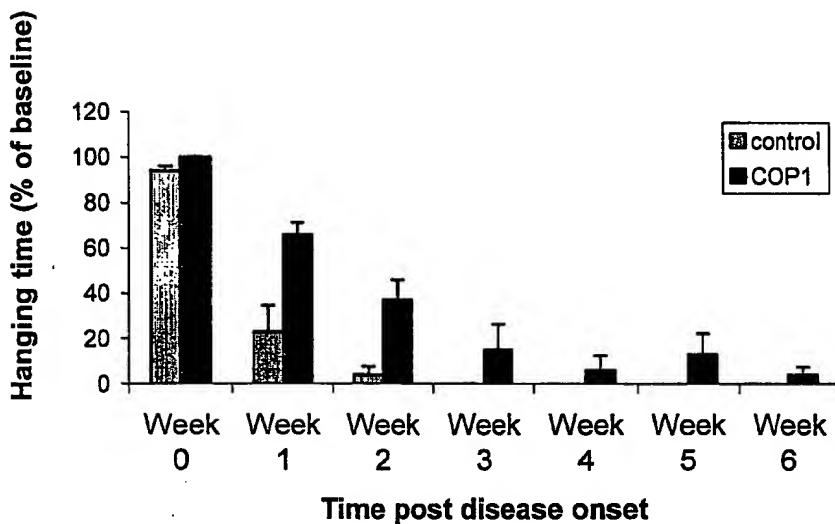


Fig. 4B

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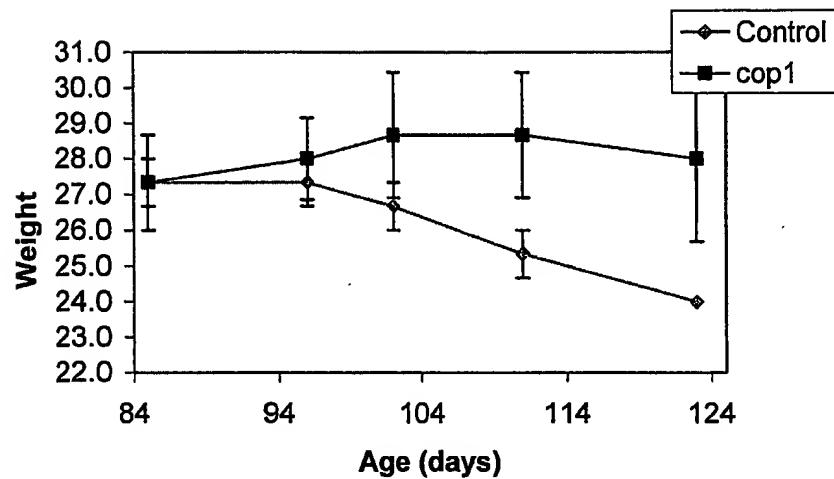


Fig. 5

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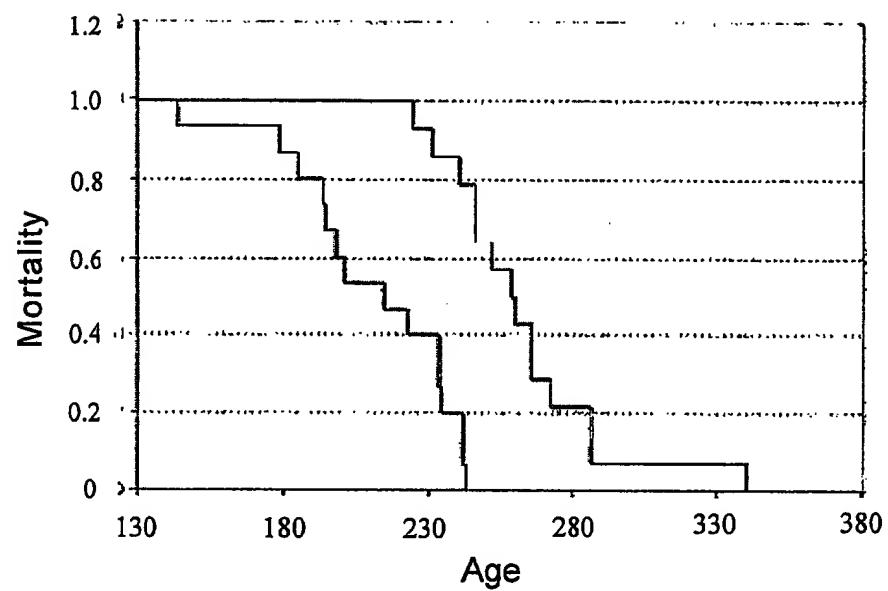


Fig. 6

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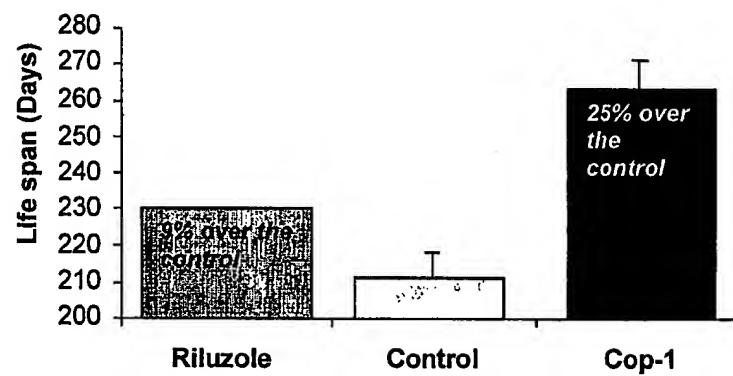


Fig. 7

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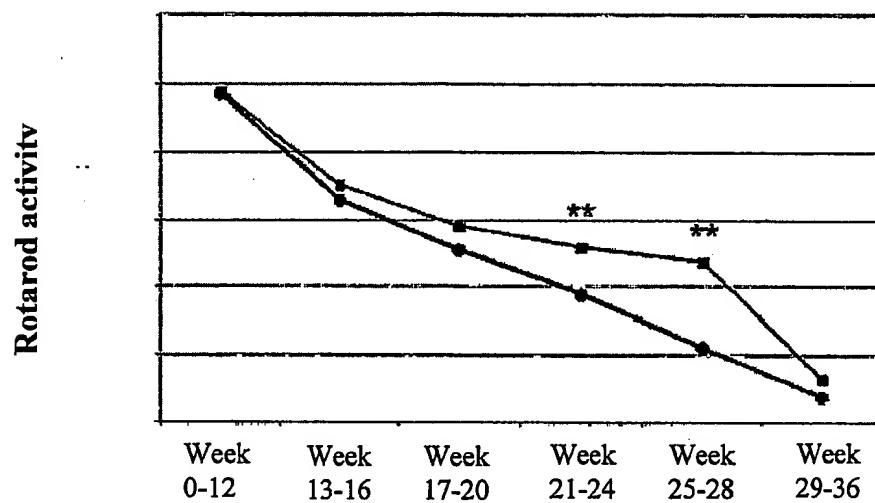


Fig. 8

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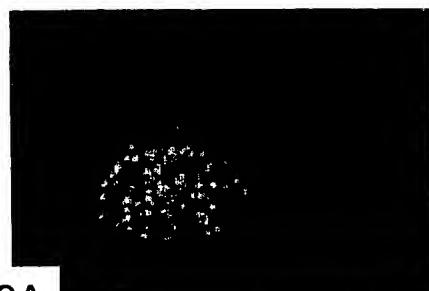


Fig. 9A



Fig. 9B



Fig. 9C



Fig. 9D

Example 4. Cop 1 immunization protects motor nerve degeneration in transgenic mutant SOD1 mice (ALS mice)

To test whether Cop 1 immunization can protect from the progression of motor neuron degeneration, ALS mice SOD1(n=3) were immunized with Cop 1 in Alum-phos when they were 75 days old and a boost was administered one week later. Then they were immunized every 30 days. A control group (n=3) of ALS mice was not immunized with Cop 1. The mice were then tested several times per week for muscle strength, by blindly testing the time of hanging on a rotating vertical rod. Each experiment lasted 5 min.

The development of muscle weakness in the mice is depicted in Figs. 4A-B. Fig. 4A depicts the average hanging time for each animal per week (results are the mean \pm SEM. As shown, two of the Cop 1-immunized animals (mice 1 and 4) exhibited longer hanging time than the non-immunized mice.

The onset of the decline in muscle strength varied among individual mice. To assess the effect of the vaccination on the rate of decline in each mouse, the muscle strength at any given time was compared to that found one week before the decline began.

Fig. 4B shows the synchronized plot of muscle strength decline in individual transgenic mice. It is clear that mice immunized with Cop-1 (black columns) showed a significantly lower rate of muscle strength decline, regardless of their strength on the day of immunization. Thus, they retained motor power for a longer period of time as compared to non-immunized animals.

The beneficial effect of Cop1 immunization is also reflected in the mice body weight. As shown in Fig. 5, as the disease progressed, the Cop-1 immunized transgenic mice also showed a slower loss of body weight. Between the age of 86 to 111 days all non-immunized transgenic mice lost 2 grams of their body weight. In contrast, in the Cop 1-immunized group, one mouse had no change and two gained 2 grams to their body weight

The immunization with Cop 1 also affected the mortality rate of the transgenic mice. With progression of the disease, the mice became paralyzed and died. Immunization with Cop 1 significantly prolonged the life of the transgenic mice:

whereas the untreated mice died 2, 3 and 4 weeks after onset of the disease, one Cop 1-immunized mice survived for 4 weeks and the other two for 7 weeks after onset of the disease (Table 4). At the time of death, the Cop 1-immunized transgenic mice were 3 weeks older, on average, than the non-immunized mice.

5

Table 4. Cop 1 immunization prolongates life span of transgenic mice overexpressing mutant human SOD-1.

	Age of death (weeks)	Death after disease onset (weeks)
Control	16.3±0.3 (n=3)	3±0.6 (n=3)
Cop 1	20±0 (n=3)	6±1 (n=3)

10

Example 5. Cop-1 treatment increases the life expectancy of ALS mice

Fourteen ALS mice, aged 60 days, were vaccinated with Cop 1 (75 µg) emulsified in CFA (Difco Laboratories, Heidelberg, Germany) containing 5 mg/ml *Mycobacterium tuberculosis*. The emulsion (total volume 200 µl) was injected into 15 the hind foot pad, and the mice were subsequently treated daily with oral Cop 1 (12.5 mg/kg/day) given in the drinking water. Mice immunized at the age of 60 days with Cop-1 and untreated control mice were observed daily and weighed weekly. Their motor activity and mortality were monitored. The age at symptom onset was determined as the age (in days) at the time of first appearance of tremors and/or 20 shaking of the limbs, or hanging (rather than splaying out) of the hind limbs when the mouse was held in the air by the tail. Loss of the righting reflex was taken to indicate the end stage of the disease. Paralysis is caused by the progressive loss of motor neurons from the spinal cord. As shown in Fig. 6, non-vaccinated controls (n = 14) became paralyzed in one or more limbs and died by the age of 211 ± 7 days (mean ± 25 SD). Cop-1-treated mice survived for 263 ± 8 days. Thus, vaccination with Cop-1 dramatically increased the life expectancy of the ALS mice (Fig. 6).

As a positive control, 15 ALS mice were given a daily dose (30 mg/kg) of Riluzole, the only drug currently given to ALS patients. As shown in Fig. 7, the Riluzole-treated mice showed an increase of 9% in survival over the control, while the Cop 1-treated mice showed an increase of 25% over the control.

5 In addition to the increase of almost 25% in life span, disease onset (manifested by motor performance) was delayed, indicating that the benefit was also expressed in the quality of life, both at pre-clinical and at clinical stages (Fig. 8). The mice were allowed to grasp and hold onto a vertical wire (2 mm diameter) with a small loop at the lower end. Normal values for each mouse were obtained by
10 assessing nightly motor activity (from 8 PM to 8 AM) between the ages of 40 and 60 days, using the rotarod apparatus (LMTB, Berlin). Their activity was recorded individually by a computerized system and assessed daily. For statistical evaluation, the rotarod activity was normalized to the mean activity of each mouse from day 40 to day 60. Data are expressed as the mean \pm standard error of the mean (SEM).
15 Rotarod testing and weight were compared by analysis of variance (ANOVA). Statistical significance was tested by one-way ANOVA followed by a post-hoc Student-Neuman-Keuls procedure with the SPSS-PC software program (SPSS, Chicago, IL). Significant differences between Cop-1-treated and untreated mice were observed at the following time periods: between days 12 and 20 ($P < .058$), between
20 days 21 and 24 ($P < .0079$), and between days 25 and 28 ($P < .0017$)

Example 6. Treatment of ALS mice with Cop 1 without adjuvant

ALS mice (15 animals per group) were divided into 11 experimental groups:

1. Non-treated mice - negative control group.
- 25 2. Riluzole-treated mice - 30mg/kg/day
3. Mice immunized with Cop 1/CFA - 75 μ g primary vaccination followed by daily oral administration of Cop 1 (12.5 mg/kg) - positive control group.
4. Mice immunized with two injections of 75 μ g Cop 1: the first one on day 45 and the second one on day 59.

5. Mice immunized as in group #4, followed by a single injection of 100 µg Cop 1 on day 87.
6. Mice immunized with two injections of 150 µg Cop 1: the first one on day 45 and the second one on day 59.
- 5 7. Mice immunized with two injections of 75 µg Cop 1: the first one on day 83 and the second one on day 97.
8. The same as group #4, with Riluzole 30mg/kg/day.
9. The same as group #5, with Riluzole 30mg/kg/day
- 10 10. The same as group #6, with Riluzole 30mg/kg/day.
- 10 11. The same as group #7, with Riluzole 30mg/kg/day.

The motor activity and body weight of the mice are monitored once a week, starting two weeks before beginning of treatment. The end stage criterion for sacrifice of the animals is defined by their inability to right themselves within 30 seconds when placed on either side on a flat surface. The decision is made by an independent 15 veterinarian as requested by the animal protocol.

Example 7. Cop-1 administration protects against motor neuron degeneration after facial nerve axotomy

Transection of the facial nerve in the adult mouse is known to cause an easily 20 visible late degeneration of 20% to 35% of the axotomized motor neurons. Therefore, axotomy of the facial nerve provides a model for ALS, which is a disease characterized by progressive motoneuron loss. The effect of immunization on the survival and function of the neurons in the facial nerve axotomy model is indicative for the potential of the treatment in attenuating neuronal loss in ALS patients.

25 Thirty-four adult female mice (12 weeks old, 20–25 g) of the C57BL/6JO1aHsd strain (Harlan Winkelmann, Borchsen, Germany) participated in this experiment. Control animals were subjected to unilateral facial nerve axotomy and were either untreated or injected with PBS emulsified in CFA. Mice in the experimental group (n = 10) were immunized with Cop 1 (total of 100 µg) or injected 30 with PBS (n = 9), both emulsified in CFA, and 7 days later were subjected to facial

nerve axotomy. Mice in a third group ($n = 8$) were axotomized without prior immunization, and mice in a fourth group ($n = 7$) were left intact.

Seven days later a facial-facial anastomosis (FFA) was created in anesthetized mice (100 mg Ketanest® plus 5 mg Rompun® per kg body weight) by microsurgical reconnection of the proximal stump to the distal stump with two 11-0 epineurial sutures (Ethicon EH 7438G, Norderstedt, Germany). The wound was closed with three 4-0 skin sutures. For assessment of recovery, facial motor neurons supplying the whiskerpad muscles were retrogradely labeled by injection of 30 μ l of 1% aqueous solution of the fluorescent retrograde tracer FluoroGold plus 2% dimethylsulfoxide (DMSO) injected into the muscles of each whisker pad. Seven days later, the mice were re-anesthetized and perfused transcardially with 0.9% NaCl followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 20 min. The brains were removed and 50- μ m-thick coronal sections were cut through the brain stems with a vibratome. Sections were observed with a Zeiss Axioskop 50 epifluorescence microscope through a custom-made HQ-Schmalband-filter set for FluoroGold (AHF Analysentechnik, Tubingen, Germany).

Eight weeks after axotomy, as shown in Figs. 9A-D and Table 5, the mean number of FluoroGold-labeled motor neurons in the mice vaccinated with Cop-1 was significantly larger than the number obtained in the group injected with PBS in CFA or in the untreated control group ($P < .05$). Treatment with Cop 1 had no effect on the number of motor neurons in the unlesioned facial nucleus. Control immunization with PBS in CFA had no protective effect.

Retrograde neuronal labeling after injection of FluoroGold into the whiskerpad showed no differences in the localization or amount of motor neurons in the intact facial nucleus between mice immunized with Cop-1 in CFA (Fig. 9A) and mice injected with PBS in CFA (Fig. 9C). In contrast, the lesioned facial nucleus, after pre-treatment of mice with Cop 1 in CFA (Fig. 9B), contained significantly more labeled motor neurons than that of the lesioned facial nucleus in control animals pre-treated with PBS in CFA (Fig. 9D). Data are presented as means \pm standard deviation (SD). Differences between the different experimental groups were detected by applying a

one-way analysis of variance (ANOVA) and a post-hoc *t* test for unpaired data with Bonferroni-Holm correction. *P* values of less than .05 were considered statistically significant.

5 **Example 8. Cop-1 administration preserves motor neuron activity after acute axotomy**

To determine whether the larger number of motor neurons found in the Cop-1-treated axotomized mice than in the controls was associated with functional improvement, whisking behavior was biometrically analyzed. Baseline parameters of
10 whisking behavior were documented in intact control mice. Under normal physiological conditions, the mystacial vibrissae are erect with anterior orientation. Their simultaneous sweeps, known as "whisking" or "sniffing", occur 5–11 times per second. The key movements of this motor activity are the protraction and retraction of the vibrissal hairs by the piloerector muscles, which are innervated by the buccal
15 branch of the facial nerve. When the facial nerve is transected, the vibrissae acquire a caudal orientation and remain motionless.

Using this model, the following parameters were evaluated: (i) protraction (forward movement of the vibrissae), measured by the rostrally opened angle between the mid-sagittal plane and the hair shaft (large protractions are represented
20 by small angle values); (ii) whisking frequency, represented by cycles of protraction and retraction (passive backward movement) per second; (iii) amplitude- the difference, in degrees, between maximal retraction and maximal protraction; (iv) angular velocity during protraction, in degrees per second; and (v) angular acceleration during protraction, in degrees per second.

25 Mice subjected to facial nerve axotomy and Cop-1 vaccination demonstrated significantly better whisking activity than the other groups of mice. This was best demonstrated by the amplitude, the angular velocity during protraction, and the angular acceleration during protraction (Table 6).

Table 5: Effect of Cop-1 vaccination on survival of motor neurons.

Group	Unlesioned facial nucleus	Lesioned facial nucleus
A: Intact mice (n = 7)	1559±135	1707±90* B,C,D
B: FFA only (n = 8)	1434±106	670±178* A,D
C: FFA after PBS/CFA injection (n = 9)	1605±142	766±104* A,D
D: FFA after vaccination with Cop-1 in CFA (n = 10)	1640±186	1172±152* A,B,C

Numerical values of the results shown in Fig. 9. Numbers (means ± SD) of facial perikarya
 5 retrogradely labeled by injection of 1% FluoroGold (30 µl) in intact mice (group A) and in
 mice that underwent FFA only (group B), FFA after injection of PBS in CFA (group C), and
 FFA after vaccination with Cop 1 in CFA (group D). Superscript letters indicate the groups
 10 with significantly different values (*P < .05). For image analysis, a CCD video camera
 system (Optronics Engineering Model DEI-470, Goleta, CA) combined with the image
 analyzing software Optimas 6.5 (Optimas, Bothell, WA) was used to manually count the
 retrogradely labeled facial motor neurons on the computer screen (42). Employing the
 fractionator principle (43), all retrogradely labeled motor neurons with visible cell nuclei
 were counted in every second section of the 50-µm-thick sections through the facial nucleus
 15 on both the operated and the unoperated side. Counting was done by two observers who
 were blinded to the treatment received by the rats.

Table 6. Effect of Cop-1 vaccination on recovery of whisking behavior after facial nerve axotomy

Group	Frequency (Hz)	Angle at maximal protraction (degrees)	Amplitude (degrees)	Angular velocity during protraction (degrees/s)	Angular acceleration during protraction (degrees/s ²)
A: Intact mice (n = 7)	6.0±1.0	65.1°±22	40°±14*B,C	627°±346*B,C,D	20084°±1508*B,C,D
B: FFA only (n = 8)	5.0±2.0	81.2°±27	11.0°±6.0*A,D	75°±43*A	1655°±1146*A
C: FFA after injection of PBS in CFA (n = 9)	5.3±1.2	64.4°±6.3	22.1°±9.9*A,D	214°±70*A	3874°±889*A
D: FFA after treatment with Cop-1 in CFA (n = 10)	5.5±0.9	68.2°±23.05	38.9°±10.6*B,C	347.8°±87.3*A	6713°±2071*A

Biometrics of normal and recovering whisking behaviour in intact mice (group A) and in mice subjected to FFA only (group B), mice subjected to FFA after injection of PBS in CFA (group C), and mice subjected to FFA after injection of Cop-1 in CFA (Group D). Values are means ± SD. Superscript letters indicate groups with significantly different values (* P < .05). The two large hairs of the C-row on each side of the face were used for biometric analysis. With the mice under light ether narcosis, all other vibrissae were clipped with small fine scissors. A digital camcorder (Panasonic NV DX-110 EG) was used to videotape the actively exploring mice for 3–5 min. Following calibration, video images of whisking behavior were sampled at 50 Hz (50 fields per sec), with the video camera shutter opened for 4 msec. Images were recorded on AY-DVM 60 EK mini-cassettes. The video sequences were slowly reviewed and 1.5-sec sequence fragments from each mouse were selected for analysis of whisking biometrics. The selection criteria used were stable position of the head, frequency of whisking, and degree of vibrissal protraction. The selected sequences were captured by a 2D/Manual Advanced Video System PEAK Motus 2000 (PEAK Performance Technologies, Englewood, CO). The spatial model consisted of three reference points (tip of the nose and the inner angles of both eyes). Each vibrissa is represented in the spatial model by two points: its base and a point on the shaft 0.5 cm from the base.

REFERENCES

Brown R.H. (1995) "Amyotrophic lateral sclerosis: recent insights from genetics and transgenic mice" *Cell* 80: 687-692

5 Bruijn L.I., Cleveland D. W. (1996) "Mechanisms of selective motor neuron death in ALS: insights from transgenic mouse models of motor neuron disease" *Neuropathol Appl Neurobiol* 22:373-387

Chou S.M. (1992) "Pathology-light microscopy of amyotrophic lateral sclerosis". In: *Handbook of Amyotrophic Lateral Sclerosis* (Smith R.A., ed.), pp. 10 133-181. New York: Marcel Dekker

Fridkis-Hareli et al. (1999) "Binding motifs of copolymer 1 to multiple sclerosis- and rheumatoid arthritis-associated HLA-DR molecules" *J Immunol.* 162(8):4697-4704

15 Fridkis-Hareli et al. (2002) "Novel synthetic amino acid copolymers that inhibit autoantigen-specific T cell responses and suppress experimental autoimmune encephalomyelitis" *J Clin Invest* 109(12):1635-1643

Gurney M.E. et al. (1994) "Motor neuron degeneration in mice that express a human Cu₁Zn superoxide dismutase" *Science* 264: 1772-1775

Hadano et al. (2001) "A gene encoding a putative GTPase regulator is mutated 20 in familial amyotrophic lateral sclerosis 2" *Nature Genetics* 29: 166-73

Hauben et al. (2000) "Autoimmune T cells as potential neuroprotective therapy for spinal cord injury" *Lancet* 355:286-287

Hirano A. (1991) "Cytopathology of amyotrophic lateral sclerosis" *Adv Neurol* 56:91-101

25 Johnson et al. (1995) "Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group," *Neurology* 1:65

Julien J.P. (2001) "Amyotrophic lateral sclerosis: unfolding the toxicity of the 30 misfolded". *Cell* 104:581-591

Kipnis et al. (2001) "Neuronal survival after CNS insult is determined by a genetically encoded autoimmune response". *J Neurosci.* 21(13):4564-71

Kong, J. and Xu, Z. (1998) "Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1" *J. Neuroscience* 18: 3241-3250

Moalem G. et al. (1999) "Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy", *Nature Medicine* 5:49-55

Mulder D.W. et al. (1986) "Familial adult motor neuron disease: amyotrophic lateral sclerosis" *Neurology* 36:511-517

Munsat T.L. et al. (1989) "Adult motor neuron disease". In: Merritt's Textbook of Neurology (Rowland L.P., ed.), pp 682-687. Philadelphia: Lea & Febiger

Pitt et al. (2000) "Glutamate excitotoxicity in a model of multiple sclerosis" *Nature Medicine* 6:67-70

Ripps M.E. et al. (1995) "Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis" *Proc Natl Acad Sci, USA* 92: 689-693

Rosen D.R. et al. (1993) "Mutations in Cu₁Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis" *Nature* 362:59-62

Rothstein J.D., et al. (1992) "Decreased glutamate transporter by the brain and spinal cord in amyotrophic lateral sclerosis". *N. Eng. J. Med.* 326: 1464-68

Turner et al. (2001) "Clinical trials in ALS: An overview. Seminars in Neurology" 21:167-175

Yang et al. (2001) "The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis", *Nature Genetics* 29: 160-165

CLAIMS:

1. A method for reducing disease progression, and/or protection of motor nerve degeneration, and/or protection from glutamate toxicity in a patient suffering from a
5 motor neurone disease (MND), which comprises immunizing said patient with a vaccine comprising an active agent selected from the group consisting of Cop 1, a Cop 1 related-peptide, a Cop 1-related polypeptide, and poly-Glu,Tyr.
2. A method according to claim 1, wherein said motor neurone disease is
10 amyotrophic lateral sclerosis (ALS).
3. A method according to claim 1, wherein said motor neurone disease is primary lateral sclerosis (PLS), progressive muscular atrophy (PMA) or progressive bulbar palsy (PBP or bulbar onset).
15
4. A method according to any one of claims 1 to 4, wherein said vaccine comprises the active agent without an adjuvant.
5. A method according to any one of claims 1 to 4, wherein said vaccine comprises
20 the active agent emulsified in an adjuvant suitable for human clinical use.
6. A method according to claim 5, wherein said adjuvant is selected from the group consisting of aluminum hydroxide, aluminum hydroxide gel, and aluminum hydroxyphosphate.
25
7. A method according to claim 6, wherein said adjuvant is amorphous aluminum hydroxyphosphate having an acidic isoelectric point and an Al:P ratio of 1:1.
8. A method according to any one of claims 1 to 7, wherein said active agent is Cop
30 1.

9. A method according to any one of claims 1 to 7, wherein said active agent is a Cop 1 related-peptide, a Cop 1-related polypeptide.
- 5 10. A method according to any one of claims 1 to 7, wherein said active agent is poly-Glu,Tyr.
11. A method according to any one of claims 1 to 10, wherein said vaccine is administered at least once a month.
- 10 12. A method according to any one of claims 1 to 10, wherein said vaccine is administered at least once every 2-3 months.
13. A method according to any one of claims 1 to 12, wherein the treatment includes administration of another drug for treatment of MND such as Riluzole.
- 15 14. A vaccine for reducing disease progression, and/or protection of motor nerve degeneration, and/or protection from glutamate toxicity in motor neurone disease (MND) patients, comprising an active agent selected from the group consisting of Cop 1, a Cop 1-related peptide, a Cop 1-related polypeptide, and poly-Glu,Tyr.
- 20 15. A vaccine according to claim 14, wherein said motor neurone disease is amyotrophic lateral sclerosis (ALS).
- 25 16. A vaccine according to claim 15, wherein said motor neurone disease is primary lateral sclerosis (PLS), progressive muscular atrophy (PMA) or progressive bulbar palsy (PBP or bulbar onset).
- 30 17. A vaccine according to any one of claims 14 to 16, wherein said vaccine comprises the active agent without an adjuvant.

18. A vaccine according to any one of claims 14 to 16, wherein said vaccine comprises the active agent emulsified in an adjuvant suitable for human clinical use.
- 5 19. A vaccine according to claim 18, wherein said adjuvant is selected from the group consisting of aluminum hydroxide, aluminum hydroxide gel, and aluminum hydroxyphosphate.
- 10 20. A vaccine according to claim 19, wherein said adjuvant is amorphous aluminum hydroxyphosphate having an acidic isoelectric point and an Al:P ratio of 1:1.
21. A vaccine according to any one of claims 14 to 20, wherein said active agent is Cop 1.
- 15 22. A vaccine according to any one of claims 14 to 20, wherein said active agent is a Cop 1-related peptide, or a Cop 1-related polypeptide.
23. A vaccine according to any one of claims 14 to 20, wherein said active agent is poly-Glu,Tyr.
- 20 24. A vaccine according to any one of claims 14 to 23, wherein said vaccine is for administration at least once a month.
- 25 25. A vaccine according to any one of claims 14 to 23, wherein said vaccine is for administration at least once every 2-3 months.
26. A vaccine according to any one of claims 14 to 25, for administration with another drug for treatment of MND such as Riluzole.

27. Use of an active agent selected from the group consisting of Cop 1, a Cop 1-related peptide, a Cop 1-related polypeptide, and poly-Glu,Tyr, for the preparation of a vaccine for reducing disease progression, and/or protection of motor nerve degeneration, and/or protection from glutamate toxicity in motor neurone disease (MND) patients.
5
28. Use according to claim 27, wherein said motor neurone disease is amyotrophic lateral sclerosis (ALS).
- 10 29. Use according to claim 27, wherein said motor neurone disease is primary lateral sclerosis (PLS), progressive muscular atrophy (PMA) or progressive bulbar palsy (PBP or bulbar onset).
- 15 30. Use according to any one of claims 27 to 29, wherein said vaccine comprises the active agent without an adjuvant.
31. Use according to any one of claims 27 to 29, wherein said vaccine comprises the active agent emulsified in an adjuvant suitable for human clinical use.
- 20 32. Use according to claim 31, wherein said adjuvant is selected from the group consisting of aluminum hydroxide, aluminum hydroxide gel, and aluminum hydroxyphosphate.
- 25 33. Use according to claim 32, wherein said adjuvant is amorphous aluminum hydroxyphosphate having an acidic isoelectric point and an Al:P ratio of 1:1.
34. Use according to any one of claims 27 to 33, wherein said active agent is Cop 1.
35. Use according to any one of claims 27 to 33, wherein said active agent is a Cop 1-related peptide or a Cop 1-related polypeptide,
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(54) Title: VACCINE AND METHOD FOR TREATMENT OF MOTOR NEURONE DISEASES

(57) Abstract: A vaccine for reducing disease progression, and/or protection of motor nerve degeneration, and/or protection from glutamate toxicity in motor neurone disease (MND), particularly amyotrophic lateral sclerosis (ALS), patients, comprising an active agent selected from the group consisting of Cop 1, a Cop 1-related peptide, a Cop 1-related polypeptide, and poly-Glu,Tyr. The active agent is preferably Cop 1 or poly-Glu,Tyr, and can be administered with or without an adjuvant.

VACCINE AND METHOD FOR TREATMENT OF MOTOR NEURONE DISEASES

5 FIELD AND BACKGROUND OF INVENTION

The present invention relates to a vaccine and methods for the treatment of Motor Neurone Diseases (MND), particularly amyotrophic lateral sclerosis (ALS).

Motor Neurone Disease (MND) is the name given to a group of related diseases affecting the motor neurones in the brain (upper motor neurons) and spinal cord (lower motor neurons). Motor neurones (or moton neurons) are the nerve cells along which the brain sends instructions, in the form of electrical impulses, to the muscles. Degeneration of the motor neurones leads to weakness and wasting of muscles. This generally occurs in arms or legs initially, some groups of muscles being affected more than others.

15 There are several classifications of MND. In most cases of MND, degeneration of both the upper and lower motor neurones occurs. This condition is called Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, and is characterized by muscle weakness, stiffness and fasciculations (muscle twitching). There are also less common forms in which a more selective degeneration 20 of either the upper motor neurones (such as Primary Lateral Sclerosis, PLS) or lower motor neurones (such as Progressive Muscular Atrophy, PMA) is observed. Progressive Bulbar Palsy (PBP or Bulbar Onset) is a version of ALS that starts with difficulties in swallowing, chewing and speaking and affects approximately 25% of ALS patients.

25 There is considerable overlap between these forms of MND. People with PMA in time develop upper motor neurone involvement and in both PMA and ALS some people may eventually experience speech and swallowing difficulties in varying degrees (bulbar onset ALS or PMA).

30 ALS, is a chronic, progressive neurodegenerative disease characterized by gradual degeneration of the nerve cells in the central nervous system (CNS) that control voluntary muscle movement. The progressive loss of motor neurons leads to

gradual skeletal muscle atrophy and to inevitable death, usually within 2-3 to ten years of the disease onset. Muscular weakness and atrophy and signs of anterior horn cell dysfunction are initially noted most often in the hands and less often in the feet. The site of onset is random, and progression is asymmetric. In the U.S.A. alone,
5 30,000 people currently have ALS and about 8,000 new cases are diagnosed each year.

ALS occurs in sporadic (SALS) and familial (FALS) forms (Mulder et al., 1986; Munsat, 1989). The primary risk factors are mostly unknown, yet 5 to 10% of all ALS patients are familial (FALS). About 20% of all familial forms were found to
10 have mutations in the gene encoding Cu/Zn superoxide dismutase type 1 on chromosome 21 (Rosen et al., 1993; Brown, 1995). SOD is an enzyme that catalyzes the conversion of superoxide anions to hydrogen peroxide, and thus SOD can protect cells against the deleterious effects of these toxic radicals. It appears that the toxicity of different SOD mutants is not due to decreased free-radical scavenging activity
15 since no correlation was found between enzymatic activity, polypeptide half-life and resistance to proteolysis with age of onset or rapidity of human disease progression (for review, see Julien, 2001). Transgenic mice expressing various SOD1 mutants developed motor neuron disease and thus constitute an accepted animal model for testing ALS and other motor neurone therapies.

Recently, a new ALS gene has been identified by two independent groups of scientists (Hadano et al., 2001; Yang et al., 2001). This new gene, called ALS2, is located on chromosome 2 and encodes for a protein named alsin. The new ALS2 gene is mutated in both people with juvenile amyotrophic lateral sclerosis (JALS), also known as ALS2, and people with juvenile primary lateral sclerosis (JPLS).
25 Mutations in different regions of the chromosome are associated with different motor neuron diseases. Specifically, a mutation in one region is found in people with ALS, while mutations in two other regions are found in people with JPLS. In the future, transgenic mice carrying these mutations will certainly constitute a further model for testing ALS therapies.

30 Numerous studies over the last decade have been devoted to understanding the etiology, prognosis and progression of the disease. No consensus has been reached,

except for admitting that it is a multi-factorial disease in terms of circumstances leading to its progression, while the etiology remains unclear.

It is evident today that many of the factors which contribute to the progression of ALS are found in many other chronic and acute neurodegenerative disorders.

5 These factors include oxidative stress, excitotoxicity, deprivation of trophic support, and ionic imbalance. Over the years attempts have been made to halt the progression of ALS, as in other chronic and acute neurodegenerative disorders, by blocking different mediators of cytotoxicity. Most of these clinical trials have had negative results (Turner et al., 2001).

10 Oxidative stress is characterized by accumulation of free radicals that can lead to motor neuron death. Free radicals damage components of the cells' membranes, proteins or genetic material by "oxidizing" them. These free radicals may be generated when the enzyme SOD malfunctions, either because of genetic mutation as occurs in some familial ALS patients or because of the chemical environment of the 15 nerve cells, or they may be generated as a result of glutamate excitotoxicity, or for some other reason. Many ALS patients take Coenzyme Z Q10 and Vitamin E in an effort to neutralize free radicals.

Glutamate is one of the most common mediators of toxicity in acute and chronic degenerative disorders (Pitt et al., 2000) like status epilepticus, cerebral 20 ischemia, traumatic brain injury, ALS, Huntington's chorea, lathyrisms and Alzheimer's disease. Glutamate is a primary excitatory neurotransmitter in the human CNS. L-glutamate is present at a majority of synapses and is capable of displaying dual activity: it plays a pivotal role in normal functioning as an essential neurotransmitter, but becomes toxic when its physiological levels are exceeded.

25 For spinal motor neurons, rapid glutamate removal following synaptic activity is accomplished by the glutamate transporter EAAT2 present in astrocytes. Decrease in EAAT2 activity and protein level was found in brain tissue of ALS patients (Rothstein et al., 1992). This could lead to increased extracellular concentration of glutamate and death of motor neurons. Clinically, the beneficial effect of Riluzole, a 30 glutamate release inhibitor, on the course of the disorder in both humans and transgenic mice, led to the approved drug treatment of ALS. However, in neutralizing

the toxic effect it is likely to interfere with the physiological functioning of glutamate as a ubiquitous CNS neurotransmitter.

The role of immune factors, cellular and molecular, in ALS has been debated over the years. It has been argued, as in many other neurodegenerative diseases, that 5 inflammation is associated with the disease propagation, and the usage of immunosuppressive drugs in ALS has been suggested. Also, in many ALS patients, a correlation was observed with the presence of anti-ganglioside antibodies, which led some researchers to suggest that ALS is an autoimmune disease. However, no conclusive evidence has been provided to support this hypothesis.

10 In the laboratory of the present inventors, it has been recently observed that under neurodegenerative conditions caused by mechanical (axotomy) or biochemical (glutamate, oxidative stress) insults, the immune system plays a critical role. Thus, it has been found that activated T cells that recognize an antigen of the nervous system (NS) promote nerve regeneration or confer neuroprotection. Reference is made to
15 PCT Publication No. WO 99/60021, the entire contents of which is hereby incorporated herein by reference. More specifically, T cells reactive to MBP were shown to be neuroprotective in rat models of partially crushed optic nerve (Moalem et al, 1999) and of spinal cord injury (Hauben et al, 2000). Until recently, it had been thought that the immune system excluded immune cells from participating in nervous
20 system repair. It was quite surprising to discover that NS-specific activated T cells could be used to promote nerve regeneration or to protect nervous system tissue from secondary degeneration which may follow damage caused by injury or disease of the CNS or peripheral nervous system (PNS).

It was further observed by the present inventors that stressful conditions in the
25 CNS harness the adaptive immune response to cope with the stress and that this response is genetically controlled. Thus, the survival rate of retinal ganglion cells in adult mice or rats after crush injury of the optic nerve or intravitreal injection of a toxic dosage of glutamate was shown to be up to two-fold higher in strains that are resistant to CNS autoimmune diseases than in susceptible strains. The difference was
30 found to be attributable to a beneficial autoimmune T cell response that was spontaneously evoked after CNS insult in the resistant but not in susceptible strains.

Thus, the survival rate of neurons as a result of such an insult is higher when T cell response directed against self is evoked, provided that it is well-regulated. In other words, it was demonstrated that a protective autoimmune response is evoked to oppose the stressful conditions so as to protect the animal from the insult
5 consequences. It was further observed that in animals with an impaired ability to regulate such a response, or in animals devoid of mature T cells (as a result of having undergone thymectomy at birth), the ability to cope with the stressful conditions is reduced. Consequently, the survival rate of neurons following CNS insult in these animals is significantly lower than in animals endowed with an effective mechanism
10 for mounting protective autoimmune T cell-mediated response (Kipnis et al., 2001).

It was then further found by the present inventors that vaccination with non-pathogenic synthetic copolymers that resemble self-proteins such as Copolymer 1 (Cop 1 or Glatiramer), a random copolymer composed of the four amino acids: tyrosine-glutamate-alanine-lysine (hereinafter "Cop 1"), and poly-Glu,Tyr
15 (hereinafter "PolyYE"), and by T cells activated thereby, after traumatic CNS insult can be used to boost the protective autoimmunity and thereby to reduce further injury-induced damage, and can further protect CNS cells from glutamate toxicity. Reference is made to our previous United States Patent Application Serial Nos. 09/756,301 and 09/765,644, both dated 22 January, 2001, herein incorporated by
20 reference in their entirety as if fully disclosed herein, corresponding to WO 01/93893, which disclose that Cop 1, Cop 1-related peptides and polypeptides and T cells activated therewith protect CNS cells from glutamate toxicity (USSN 09/756,301) and prevent or inhibit neuronal degeneration or promote nerve regeneration in the CNS or PNS (USSN 09/765,644). Reference is further made to our previous United
25 States Patent Application Serial No. 09/893,344 dated 28 June, 2001, herein incorporated by reference in its entirety as if fully disclosed herein, which discloses that the copolymer poly-Glu⁵⁰Tyr⁵⁰, formerly called polyGT and also designated PolyYE, and T cells activated therewith, protect CNS cells from glutamate toxicity and also prevent or inhibit neuronal degeneration or promote nerve regeneration in the
30 CNS or PNS. Specifically, it was shown in said applications that in optic nerve fibers,

the number of surviving retinal ganglion cells was significantly higher in the Cop 1-immunized or poly-Glu,Tyr-immunized mice than in the mice injected with PBS.

The sole drug approved and currently available for treatment of ALS is Riluzole (2-amino-6-(trifluoromethoxy)benzothiazole), a putative blocker of glutamate release, which appears to have some spasm-reducing effects in this condition, possibly through inhibition of glutamatergic transmission in the CNS. It is administered orally in the form of tablets. Riluzole does not cure the disease or improve symptoms. It exerts a modest to significant effect in ALS patients by elongating their life span for about 3 months, but does not improve muscular strength or neurologic function.

It would be highly desirable to provide further medicaments for the treatment of motor neuron diseases, including ALS.

Citation or identification of any reference in this section or any other part of this application shall not be construed as an admission that such reference is available as prior art to the invention.

SUMMARY OF THE INVENTION

It has now been found, in accordance with the present invention, that immunization with Cop 1 can protect transgenic mice overexpressing human SOD1 and mice after facial nerve axotomy, both models for ALS, from motor neuron degeneration. This and the fact that both Cop 1 and PolyYE are effective in protecting retinal ganglion cells from glutamate toxicity, indicates the suitability of these copolymers for the treatment of motor neurone diseases, particularly ALS.

The present invention thus relates, in one aspect, to a method for reducing disease progression, for protection of motor neuron degeneration and/or for protection from glutamate toxicity in a patient suffering from a motor neurone disease (MND), which comprises immunizing said patient with a vaccine comprising an active agent selected from the group consisting of Cop 1, a Cop 1-related peptide, a Cop 1-related polypeptide, and PolyYE.

The motor neurone disease (MND) is any disease affecting the motor neurones in the brain and spinal cord and includes amyotrophic lateral sclerosis (ALS), both

familial (FALS) and sporadic (SALS) ALS, primary lateral sclerosis (PLS), progressive muscular atrophy (PMA), progressive bulbar palsy (PBP or bulbar onset), and combined forms thereof such as bulbar onset ALS and bulbar onset PMA.

In one embodiment, the method of the invention includes treatment also with
5 Riluzole or any other drug suitable for treatment of MND, particularly ALS.

In another aspect, the present invention provides a vaccine for reducing disease progression, for protection of motor nerve degeneration and/or for protection from glutamate toxicity in a motor neurone disease (MND), particularly ALS, comprising an active agent selected from the group consisting of Cop 1, a Cop 1-related peptide, a Cop 1-related polypeptide, and poly-Glu,Tyr.
10

In a further aspect, the present invention relates to the use of an active agent selected from the group consisting of Cop 1, a Cop 1-related peptide, a Cop 1-related polypeptide, and poly-Glu,Tyr, for the manufacture of a vaccine for reducing disease progression, for protection of motor nerve degeneration and/or for protection from glutamate toxicity in motor neurone disease (MND), particularly ALS.
15

The active agent may be administered without any adjuvant or it may be emulsified in an adjuvant suitable for human clinical use. The adjuvant suitable for human clinical use is selected from aluminum hydroxide, aluminum hydroxide gel, and aluminum hydroxyphosphate. In a preferred embodiment, the vaccine adjuvant is amorphous aluminum hydroxyphosphate having an acidic isoelectric point and an Al:P ratio of 1:1 (herein referred to as Alum-phos).
20

In one preferred embodiment, the active agent of the vaccine of the invention is Cop 1. In another preferred embodiment, the active agent is poly-Glu,Tyr.

In addition, the vaccine may be administered in a regimen that includes
25 administration of Riluzole or another drug suitable for treatment of ALS.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 shows that immunization with Cop 1 or PolyYE without adjuvant protects mice retinal ganglion cells (RGCs) from glutamate toxicity.

30 **Figs. 2A-B** show that immunization with Cop 1 (2A) or PolyYE (2B) in adjuvant (CFA) protects mice RGCs from glutamate toxicity.

Figs. 3A-B show the effect of immunization with PolyYE (Fig. 3A) or Cop 1 (Fig. 3B) on RGC survival in the glaucoma intraocular pressure (IOP) model.

Figs. 4A-4B depict results of muscle strength test carried out with transgenic mice overexpressing human mutant SOD1 (hereinafter "ALS mice"). Fig. 4A shows the average hanging time (seconds) on a rotating vertical rod per week of ALS mice immunized with Cop 1 emulsified in Alum-phos (mice 1, 2 and 4) and of non-immunized transgenic mice (mice 3, 5 and 6). Fig. 4B depicts the average hanging time (% of baseline) of 3 ALS mice immunized with Cop 1 in Alum-phos (black columns) as compared to that of 3 transgenic non-immunized mice (control, gray columns). To compare the rate of disease progression, all the animals were synchronized to the time of onset of muscle weakness (time 0), normalizing each animal hanging time to its own baseline time before the disease onset (baseline time - 100%). The figure depicts the average \pm SEM hanging time per each group, for the following weeks of disease progression.

Fig. 5 shows the preservation of body weight in ALS mice immunized with Cop 1 in Alum-phos (black squares) as compared to non-immunized mice (gray diamonds).

Fig. 6 is a graph showing life expectancy in ALS mice immunized with Cop-1 in CFA. Paralysis is caused by the progressive loss of motor neurons from the spinal cord. Non-vaccinated controls ($n = 15$) became paralyzed in one or more limbs and died by the age of 211 ± 7 days (mean \pm SD). Cop 1-treated mice survived for 263 ± 8 days.

Fig. 7 shows life expectancy in ALS mice immunized with Cop-1 in CFA and ALS mice-treated with Riluzole. Riluzole-treated and Cop 1-immunized ALS mice showed an increase of 9% and 25%, respectively, over the non-vaccinated control mice.

Fig. 8 shows average rotatory activity measured at the indicated time points in Cop 1-treated and untreated ALS mice. The mice were allowed to grasp and hold onto a vertical wire (2 mm diameter) with a small loop at the lower end. Their activity was recorded individually by a computerized system and assessed daily. For statistical evaluation, the rotarod activity was normalized to the mean activity of each

mouse from day 40 to day 60. Data are expressed as the mean \pm standard error of the mean (SEM). Significant differences between treated and untreated mice were observed at the following time periods: between days 12 and 20 ($P < .058$), between days 21 and 24 ($P < .0079$), and between days 25 and 28 ($P < .0017$).

5 Figs. 9A-D shows rescue of motor neurons by Cop 1 administered to mice after facial nerve axotomy. Eight weeks after axotomy the number of FluoroGold-labeled motor neurons in the brain stems of mice vaccinated with Cop-1 (Fig. 9D) was significantly larger than the number obtained in the group injected with PBS in CFA (Fig. 9B). Treatment with Cop-1 had no effect on the number of motor neurons
10 in the unlesioned facial nucleus (Figs. 9A, 9C). Control immunization with PBS in CFA had no protective effect.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a vaccine and a method for reducing disease progression, for protection of motor nerve degeneration, for prolonging life span and improving quality of life, and/or for protection from glutamate toxicity in a patient suffering from MND, particularly ALS, which comprises immunizing said patient with a vaccine comprising an active agent selected from the group consisting of Cop 1, a Cop 1-related peptide, a Cop 1-related polypeptide, or PolyYE, either without adjuvant or emulsified in an adjuvant suitable for human clinical use.
15
20

As used herein, the terms "motor neurons" and "moton neurons", the terms "PolyYE" and "poly-Glu,Tyr", and the terms "Cop 1" and Copolymer 1", are each used interchangeably.

For the purpose of the present invention, "Cop 1 or a Cop 1-related peptide or polypeptide" is intended to include any peptide or polypeptide, including a random copolymer, that cross-reacts functionally with myelin basic protein (MBP) and is able to compete with MBP on the MHC class II in the antigen presentation.
25

The vaccine of the invention may comprise as active agent a random copolymer comprising a suitable quantity of a positively charged amino acid such as lysine or arginine, in combination with a negatively charged amino acid (preferably in a lesser quantity) such as glutamic acid or aspartic acid, optionally in combination
30

with a non-charged neutral amino acid such as alanine or glycine, serving as a filler, and optionally with an amino acid adapted to confer on the copolymer immunogenic properties, such as an aromatic amino acid like tyrosine or tryptophan. Such vaccines may include any of those copolymers disclosed in WO 00/05250, the entire contents
5 of which being hereby incorporated herein by reference.

More specifically, the vaccine for use in the present invention comprises at least one copolymer selected from the group consisting of random copolymers comprising one amino acid selected from each of at least three of the following groups: (a) lysine and arginine; (b) glutamic acid and aspartic acid; (c)
10 alanine and glycine; and (d) tyrosine and tryptophan.

The copolymers for use in the present invention can be composed of L- or D-amino acids or mixtures thereof. As is known by those of skill in the art, L-amino acids occur in most natural proteins. However, D-amino acids are commercially available and can be substituted for some or all of the amino acids used to make the
15 terpolymers and other copolymers used in the present invention. The present invention contemplates the use of copolymers containing both D- and L-amino acids, as well as copolymers consisting essentially of either L- or D-amino acids.

In one embodiment of the invention, the copolymer contains four different amino acids, each from a different one of the groups (a) to (d). A preferred
20 copolymer according to this embodiment comprises in combination alanine, glutamic acid, lysine, and tyrosine, of net overall positive electrical charge and of a molecular weight of about 2,000 - 40,000 Da, preferably of about 2,000 - 13,000 Da, and is most preferably Copolymer 1 of average molecular weight of about 4,700 - 13,000 Da. Preferred molecular weight ranges and processes for making a preferred form of Cop
25 1 are described in U.S. Patent No. 5,800,808, the entire contents of which being hereby incorporated in the entirety. It is clear that this is given by way of example only, and that the vaccine can be varied both with respect to the constituents and relative proportions of the constituents if the above general criteria are adhered to. Thus, the copolymer may be a polypeptide from about 15 to about 100, preferably
30 from about 40 to about 80, amino acids in length, and is preferably the copolymer having the generic name glatiramer acetate.

In another embodiment, the copolymer contains three different amino acids each from a different one of three groups of the groups (a) to (d). These copolymers are herein referred to as terpolymers.

In one embodiment, the terpolymers for use in the present invention contain 5 tyrosine, alanine, and lysine, hereinafter designated YAK. The average molar fraction of the amino acids in these terpolymers can vary. For example, tyrosine can be present in a mole fraction of about 0.005-0.250; alanine can be present in a mole fraction of about 0.3 - 0.6; and lysine can be present in a mole fraction of about 0.1-0.5. The average molecular weight is between 2,000 - 40,000 Da, and preferably 10 between about 3,000 - 35,000 Da. In a more preferred embodiment, the average molecular weight is about 5,000 - 25,000 Da. It is possible to substitute arginine for lysine, glycine for alanine, and/or tryptophan for tyrosine.

In another embodiment, the terpolymers for use in the present invention contain tyrosine, glutamic acid, and lysine, hereinafter designated YEK. The average 15 molar fraction of the amino acids in these terpolymers can vary: glutamic acid can be present in a mole fraction of about 0.005 - 0.300, tyrosine can be present in a mole fraction of about 0.005 - 0.250, and lysine can be present in a mole fraction of about 0.3 - 0.7. The average molecular weight is between 2,000 - 40,000 Da, and preferably between about 3,000 - 35,000 Da. In a more preferred embodiment, the 20 average molecular weight is about 5,000 - 25,000 Da. It is possible to substitute aspartic acid for glutamic acid, arginine for lysine, and/or tryptophan for tyrosine.

In another embodiment the terpolymers for use in the present invention contain lysine, glutamic acid, and alanine, hereinafter designated KEA. The average 25 molar fraction of the amino acids in these polypeptides can also vary. For example, glutamic acid can be present in a mole fraction of about 0.005 - 0.300, alanine can be present in a mole fraction of about 0.005 - 0.600, lysine can be present in a mole fraction of about 0.2 - 0.7. The average molecular weight is between 2,000 - 40,000 Da, and preferably between about 3,000 - 35,000 Da. In a more preferred embodiment, the average molecular weight is about 5,000 - 25,000 Da. It is possible 30 to substitute aspartic acid for glutamic acid, glycine for alanine, and/or arginine for lysine.

In another embodiment, the terpolymers for use in the present invention contain tyrosine, glutamic acid, and alanine, hereinafter designated YEA. The average molar fraction of the amino acids in these polypeptides can vary. For example, tyrosine can be present in a mole fraction of about 0.005 - 0.250, glutamic acid can be present in a mole fraction of about 0.005 - 0.300, and alanine can be present in a mole fraction of about 0.005 - 0.800. The average molecular weight is between 2,000 - 40,000 Da, and preferably between about 3,000 - 35,000 Da. In a more preferred embodiment, the average molecular weight is about 5,000 - 25,000 Da. It is possible to substitute tryptophan for tyrosine, aspartic acid for glutamic acid, and/or glycine for alanine.

In a more preferred embodiment, the mole fraction of amino acids of the terpolymers is about what is preferred for Copolymer 1. The mole fraction of amino acids in Copolymer 1 is glutamic acid about 0.14, alanine about 0.43, tyrosine about 0.10, and lysine about 0.34. The most preferred average molecular weight for Copolymer 1 is between about 5,000 - 9,000 Da. The activity of Copolymer 1 for the vaccine disclosed herein is expected to remain if one or more of the following substitutions is made: aspartic acid for glutamic acid, glycine for alanine, arginine for lysine, and tryptophan for tyrosine.

The molar ratios of the monomers of the more preferred terpolymer of glutamic acid, alanine, and tyrosine, or YEA, is about 0.21 to about 0.65 to about 0.14.

The molar ratios of the monomers of the more preferred terpolymer of glutamic acid, alanine and lysine, or KEA, is about 0.15 to about 0.48 to about 0.36.

The molar ratios of the monomers of the more preferred terpolymer of glutamic acid, tyrosine, and lysine, or YEK, is about 0.26 to about 0.16 to about 0.58.

The molar ratios of the monomers of the more preferred terpolymer of tyrosine, alanine and lysine, or YAK, is about 0.10 to about 0.54 to about 0.35.

The terpolymers can be made by any procedure available to one of skill in the art. For example, the terpolymers can be made under condensation conditions using the desired molar ratio of amino acids in solution, or by solid phase synthetic procedures. Condensation conditions include the proper temperature, pH, and solvent

conditions for condensing the carboxyl group of one amino acid with the amino group of another amino acid to form a peptide bond. Condensing agents, for example dicyclohexyl-carbodiimide, can be used to facilitate the formation of the peptide bond. Blocking groups can be used to protect functional groups, such as the side chain moieties and some of the amino or carboxyl groups against undesired side reactions.

For example, the process disclosed in U.S. Patent 3,849,650, can be used wherein the N-carboxyanhydrides of tyrosine, alanine, γ -benzyl glutamate and N ϵ -trifluoroacetyl-lysine are polymerized at ambient temperatures in anhydrous dioxane with diethylamine as an initiator. The γ -carboxyl group of the glutamic acid can be deblocked by hydrogen bromide in glacial acetic acid. The trifluoroacetyl groups are removed from lysine by 1 molar piperidine. One of skill in the art readily understands that the process can be adjusted to make peptides and polypeptides containing the desired amino acids, that is, three of the four amino acids in Copolymer 1, by selectively eliminating the reactions that relate to any one of glutamic acid, alanine, tyrosine, or lysine. For purposes of this application, the terms "ambient temperature" and "room temperature" mean a temperature ranging from about 20 to about 26°C.

The molecular weight of the terpolymers can be adjusted during polypeptide synthesis or after the terpolymers have been made. To adjust the molecular weight during polypeptide synthesis, the synthetic conditions or the amounts of amino acids are adjusted so that synthesis stops when the polypeptide reaches the approximate length which is desired. After synthesis, polypeptides with the desired molecular weight can be obtained by any available size selection procedure, such as chromatography of the polypeptides on a molecular weight sizing column or gel, and collection of the molecular weight ranges desired. The present polypeptides can also be partially hydrolyzed to remove high molecular weight species, for example, by acid or enzymatic hydrolysis, and then purified to remove the acid or enzymes.

In one embodiment, the terpolymers with a desired molecular weight may be prepared by a process which includes reacting a protected polypeptide with hydrobromic acid to form a trifluoroacetyl-polypeptide having the desired molecular

weight profile. The reaction is performed for a time and at a temperature which is predetermined by one or more test reactions. During the test reaction, the time and temperature are varied and the molecular weight range of a given batch of test polypeptides is determined. The test conditions which provide the optimal molecular 5 weight range for that batch of polypeptides are used for the batch. Thus, a trifluoroacetyl-polypeptide having the desired molecular weight profile can be produced by a process which includes reacting the protected polypeptide with hydrobromic acid for a time and at a temperature predetermined by test reaction. The trifluoroacetyl-polypeptide with the desired molecular weight profile is then further 10 treated with an aqueous piperidine solution to form a low toxicity polypeptide having the desired molecular weight.

In a preferred embodiment, a test sample of protected polypeptide from a given batch is reacted with hydrobromic acid for about 10-50 hours at a temperature of about 20-28°C. The best conditions for that batch are determined by running 15 several test reactions. For example, in one embodiment, the protected polypeptide is reacted with hydrobromic acid for about 17 hours at a temperature of about 26°C.

As binding motifs of Cop 1 to MS-associated HLA-DR molecules are known (Fridkis-Hareli et al, 1999), polypeptides of fixed sequence can readily be prepared and tested for binding to the peptide binding groove of the HLA-DR molecules as 20 described in the Fridkis-Hareli et al (1999) publication. Examples of such peptides are those disclosed in WO 005249, the entire contents of which being hereby incorporated herein by reference. Thirty-two of the peptides specifically disclosed in said application are reproduced in Table 1, hereinbelow. Such peptides and other similar peptides would be expected to have similar activity as Cop 1. Such peptides, 25 and other similar peptides, are also considered to be within the definition of Cop 1-related peptides or polypeptides and their use is considered to be part of the present invention.

The definition of "Cop 1 related-polypeptide" according to the invention is meant to encompass other synthetic amino acid copolymers such as the random four-amino acid copolymers described by Fridkis-Hareli et al., 2002, as candidates for 30 treatment of multiple sclerosis, namely copolymers (14-, 35- and 50-mers) containing

the amino acids phenylalanine, glutamic acid, alanine and lysine (poly FEAK), or tyrosine, phenylalanine, alanine and lysine (poly YFAK), and any other similar copolymer to be discovered that can be considered a universal antigen similar to Cop 1 and polyYE.

5

Table 1

SEQ ID NO.	Peptide Sequence
1	AAAYAAAAAAKAAAAA
2	AEKYAAAAAAKAAAAA
3	AKEYAAAAAAKAAAAA
4	AKKYAAAAAAKAAAAA
5	AEAYAAAAAAKAAAAA
6	KEAYAAAAAAKAAAAA
7	AEEYAAAAAAKAAAAA
8	AAEYAAAAAAKAAAAA
9	EKAYAAAAAAKAAAAA
10	AAKYEAaaaaAKAAAA
11	AAKYAEAAAAKAAAA
12	EAAYAAAAAAKAAAAA
13	EKKYAAAAAAKAAAAA
14	EAKYAAAAAAKAAAAA
15	AEKYAAAAAAAAAAAAA
16	AKYEAAAAAAAAAAAAA
17	AKKYEAaaaaaaaaaa
18	AKKYAEaaaaaaaaaa
19	AEAYKAaaaaaaaaaa
20	KEAYAAAAAAAAAAAAA
21	AEEYKAaaaaaaaaaa
22	AAEYKAaaaaaaaaaa
23	EKAYAAAAAAAAAAAAA
24	AAKYEAaaaaaaaaaa
25	EKKYAAAAAAAAAAAAA
26	EAKYAAAAAAAAAAAAA
27	AEYAKAAAAAAAAAAA
28	AEKAYAAAAAAAAAAA
29	EKYAAAAAAAAAAAAA
30	AYKAEEaaaaaaaaaa
31	AKYAEaaaaaaaaaaa
32	

According to the present invention, the preferred copolymer for use in the vaccine of the invention is Copolymer 1, herein referred to also as Cop 1, most preferably in the form of its acetate salt known under the generic name Glatiramer acetate. Glatiramer acetate has been approved in several countries for the treatment 5 of multiple sclerosis (MS) under the trade name, COPAXONE® (a trademark of Teva Pharmaceuticals Ltd., Petah Tikva, Israel). Several clinical trials demonstrated that Cop 1 is well tolerated with only minor side reactions which were mostly mild reactions at the injection site (Johnson et al, 1995).

As mentioned before, mutations in the SOD1 gene are one genetic cause for 10 familial ALS (Rosen et al., 1993; Brown, 1995). Several mouse models that express the mutated SOD1 genes develop motor neuron degeneration similar to that in humans (Gurney et al., 1994; Rипps et al., 1995; Kong and Xu, 1998). The initial characterization of these mouse lines has proven that a dominant gain of an adverse property by the mutated enzymes causes motor neuron degeneration (for review, see 15 Bruijn and Cleveland, 1996). In addition, these analyses confirmed numerous pathological features that have been observed in humans (Hirano, 1991; Chou, 1992). Understanding this mutation, called SOD1 alteration, yielded an accepted animal model (ALS mice) for testing therapies for familial ALS. Since SOD1-related familial ALS and sporadic ALS (which accounts for 90% of all ALS cases) have 20 similar symptoms and pathological features, the transgenic mouse carrying a mutated SOD1 gene is an accepted animal model for testing therapies for both familial and sporadic ALS forms, and is the model used by the ALS Therapy Development Foundation (ALS-TDF). ALS mice develop a motor disease that closely resembles ALS. The motor dysfunction eventually causes their death.

According to the present invention, ALS mice which were immunized with a 25 vaccine of Cop 1 emulsified in CFA or in an adjuvant suitable for human use were shown to be protected from motor nerve degeneration, in spite of the oxidative stress conditions created by the overexpression of SOD. Thus, vaccination with the “universal” weak self-reactive antigen Cop 1 in CFA prolonged by 52 days the life 30 span of ALS mice (mean±SD, 263±8 days, n=14) compared to untreated matched controls (211±7 days; n=15; P<.0001). The vaccination significantly improved motor

activity in the clinical and pre-clinical stages. In addition, vaccination with Cop 1 also prevented acute motor neuron degeneration after facial nerve axotomy: almost 200% more motor neurons survived in vaccinated mice than in axotomized controls ($P<.05$). These results suggest that the concept of autoimmunity as protective can be
5 extended to include motor neuron diseases. They also have potentially dramatic clinical implications.

The adjuvants used for the immunization according to the invention are aluminum-based adjuvants. More commonly used in vaccines containing virus-derived antigens such as hepatitis B surface antigen or Haemophilus influenza type b
10 capsular polysaccharide, these adjuvants are for the first time used together with synthetic copolymers, particularly with Cop 1.

The dosage of Cop 1 or PolyYE to be administered will be determined by the physician according to the age of the patient and stage of the disease and may be chosen from a range of 10-80 mg, although any other suitable dosage is encompassed
15 by the invention. The administration may be made at least once a month or at least once every 2 or 3 months, or less frequently, but any other suitable interval between the immunizations is envisaged by the invention according to the condition of the patient.

The vaccine of the invention may be administered by any suitable mode of
20 administration, including orally, intramuscularly, subcutaneously and intradermally, with or without adjuvant.

When administered together with Riluzole or any other drug suitable for treatment of MND, particularly ALS, the additional drug is administered at the same day of vaccination, and daily thereafter, according to the manufacturer's instructions,
25 with no association to the vaccine regimen. For example, the daily dose of Riluzole is 100 mg.

The following examples illustrate certain features of the present invention but are not intended to limit the scope of the present invention.

EXAMPLES

Materials and Methods

Animals. Mice of the C57BL/6J strain, aged 8-13 weeks, were supplied by the Animal Breeding Center of The Weizmann Institute of Science (Rehovot, Israel).

5 Prior to their use in the experiments, the mice were anesthetized by intraperitoneal administration of 80 mg/kg ketamine and 16 mg/kg xylazine. Transgenic mice overexpressing the defective human mutant SOD1 allele containing the Gly93→Ala (G93A) gene (B6SJL-TgN (SOD1-G93A)1Gur (herein "ALS mice") were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animals were
10 handled according to the regulations formulated by the Institutional Animal Care and Use Committee (IACUC).

Materials. Cop 1 (median MW: 7,200 dalton) was from Teva Pharmaceuticals Ltd. (Petah Tikva, Israel). Aluminum hydroxyphosphate gel (REHYDRAPHOS™

15 Vaccine Adjuvant, herein Alum-phos) was purchased from Reheis (NJ, USA). Complete Freund's adjuvant containing 0.5 mg/ml *Mycobacterium tuberculosis* (CFA) was purchased from Difco (Detroit, Michigan, USA), if not stated otherwise.

Immunization. Mice were immunized with Cop 1 emulsified in CFA or in Cop 1-
20 Alum-phos (100 µg in a total volume of 100 µl). Alum-phos was mixed vigorously with Cop 1 in a ratio of 1:4. Each vaccine was injected subcutaneously (SC) at one site in the flank of the mice. Control mice were injected with mannitol in either CFA or in Alum-phos.

25 **Glutamate injection.** The right eye of an anesthetized C57B BL/6J mouse was punctured with a 27-gauge needle in the upper part of the sclera, and a 10-µl Hamilton syringe with a 30-gauge needle was inserted as far as the vitreal body. Mice were injected with a total volume of 1 µl (200 nmol) of L-glutamate dissolved in saline.

Labeling of retinal ganglion cells (RGC) in mice. RGCs were labeled 72 hours before the end of the experiment. Mice were anesthetized and placed in a stereotactic device. The skull was exposed and kept dry and clean. The bregma was identified and marked. The designated point of injection was at a depth of 2 mm from the brain

5 surface, 2.92 mm behind the bregma in the anteroposterior axis and 0.5 mm lateral to the midline. A window was drilled in the scalp above the designated coordinates in the right and left hemispheres. The neurotracer dye FluoroGold (5% solution in saline; Fluorochrome, Denver, CO) was then applied (1 μ l, at a rate of 0.5 μ l/min in each hemisphere) using a Hamilton syringe, and the skin over the wound was sutured.

10 Retrograde uptake of the dye provides a marker of the living cells.

Assessment of RGC survival in mice. Mice were given a lethal dose of pentobarbitone (170 mg/kg). Their eyes were enucleated and the retinas were detached and prepared as flattened whole mounts in paraformaldehyde (4% in PBS).

15 Labeled cells from 4–6 selected fields of identical size (0.7 mm^2) were counted. The selected fields were located at approximately the same distance from the optic disk (0.3 mm) to overcome the variation in RGC density as a function of distance from the optic disk. Fields were counted under the fluorescence microscope (magnification $\times 800$) by observers blinded to the treatment received by the mouse. The average

20 number of RGCs per field in each retina was calculated.

Amyotrophic lateral sclerosis model. Three ALS mice, aged 75 days, were vaccinated with Cop-1 emulsified in Alum-phos (100 μ g Cop-1 in a total volume of 100 μ l, one subcutaneous injection in the flank). The mice were given a booster

25 injection a week later and monthly injections thereafter. Three additional transgenic mice were not immunized and served as a control for spontaneous progression of the disease. The muscle strength was evaluated by blindly testing the time of hanging of each mouse on a rotating vertical rod. Since the maximal time that most of the animals were able to hang on the rotating rod was 5 minutes, each experiment was

30 continued up to 5 minutes.

Muscle strength test. The test was performed as previously described (Kong and Xu, 1998). Mice were allowed to grasp and hold onto a vertical wire (2 mm in diameter) with a small loop at the lower end. A vertical wire allows mice to use both fore- and hindlimbs to grab onto the wire. The wire was maintained in a vertically oriented circular motion (the circle radius was 10 cm) at 24 rpm. The time that the mouse was able to hang onto the wire was recorded with a timer. Because most mice fell within 5 min, the testing was cut off at 5 min. Mice were usually tested once a week and testing continued until they could no longer hang onto the wire.

10

Data analysis. Survival data were analyzed by the Mantel-Cox test or Cox's proportional hazards regression analysis. Statistical significance was tested by one-way ANOVA, followed by a post-hoc Student-Neuman-Keuls procedure with the SPSS-PC software program (SPSS, Chicago, IL).

15

Example 1. Neuronal protection against glutamate toxicity by active vaccination with Cop 1 emulsified in Alum-phos.

It was first examined whether glutamate-induced toxicity can be blocked by active vaccination with Cop 1 emulsified in CFA or in Alum-phos. CFA is an adjuvant not approved for human use and is used frequently only in laboratory animal experiments. Alum-phos and other aluminum hydroxide-based adjuvants have received FDA and other authorities approval and are extensively used in veterinary and human vaccines.

Cop 1 emulsified either in CFA or in Alum-phos (100 µg Cop 1 in total volume of 100 µl) was injected subcutaneously at one site in the flank of C57BL/6J mice, and seven days later glutamate (200 nmol) was injected into the vitreal body of the mice. After seven days the surviving RGCs were counted. The survival of RGCs following glutamate toxicity without any prior immunization was taken as 100%.

As shown in Table 2, pre-immunization with Cop-1 either in CFA or in Alum-phos seven days before glutamate injection yielded a significant protection of retinal

ganglion cells against glutamate toxicity, but the protection with Cop 1 emulsifie in Alum-phos was significantly higher than in CFA.

**Table 2: Neuronal protection against glutamate toxicity by active vaccination
5 with Cop-1 in CFA or in Alum-phos**

		RGC survival (% of non-immunized)	
		Control Immunization	Cop-1
CFA	$98 \pm 3\% ; n = 11$	$118 \pm 8.2\% *; n = 9$	
Alum-phos	$108 \pm 11\% *; n = 8$	$135 \pm 7\% *; n = 8$	

*p < 0.05; 2-tailed Student's t-test.

**Example 2. Neuronal protection against glutamate toxicity by vaccination with
Cop 1 or PolyYE without or with adjuvant**

10 Glutamate toxicity is one of the risk factors in ALS neurodegeneration. To examine the efficacy of immunization with Cop 1 and PolyYE without adjuvant to protect the neurons from glutamate toxicity, the retina of C57BL mice were exposed to excess amount of glutamate. The C57BL mice were divided into 4 experimental groups:

15 1. Animals that were not immunized – negative control, n=9
 2. Animals that were immunized with 25 µg PolyYE per mice, n=10
 3. Animals that were immunized with 225 µg PolyYE per mice, n=10
 4. Animals that were immunized with 75 µg Cop1 per mice, n=7

The treated groups were immunized with PolyYE or Cop 1 dissolved in 100 µl PBS 20 7 days prior to intraocular glutamate injection. The number of RGCs that survived 7 days after exposure to elevated level of glutamate was counted and calculated as percentage of normal eyes. The results are shown in Fig. 1. RGC survival in all the treated groups (groups 2-4) was significantly (p<0.001 t-test) higher than the negative control group.

25 In additional experiments, C57Bl mice were treated with Cop 1 (100 µg) emulsified in Alum-phos (n=8) or with Alum-phos alone (n=8) or PolyYE (100 µg)

emulsified in CFA (n=24) or with adjuvant alone (negative control) (n=27) (100 µl), 7 days prior to intraocular glutamate injection. The number of RGCs that survived 7 days after exposure to elevated level of glutamate was counted. Protection was calculated as percentage of RGC that survived out of the total RGC loss in the non-treated group.. The results are shown in Figs. 2A-B. RGC survival in the Cop 1-treated group (Fig. 2A) and the PolyYE-treated group (Fig. 2B) was significantly higher than the negative control groups that received adjuvant alone.

Cop 1 of high molecular weight sizes (median MW: 12,600, 15,500, and 22,000 dalton) are tested in the glutamate toxicity model. The efficacy in evoking specific neuroprotective response is determined in the model of acute glutamate toxicity in RGCs as described above. C57BL/6 mice (total of 5 groups per experiment, 10 animals per group) are immunized 14 days before intraocular injection of glutamate (200 nmol), and RGC survival is examined 7 days after glutamate injection. Three doses of Cop 1 of each MW is tested and compared to negative control (glutamate only) and positive control (75µg Cop 1 of MW 7,200 d, 7 days prior to glutamate toxicity).

Example 3. Neuroprotective effect of vaccination with Cop 1 and poly-YE in the glaucoma model

Glaucoma is a chronic neurodegenerative disease with progressive loss of visual neurons that eventually leads to blindness. Increased intraocular pressure (IOP) is considered the major risk factor and believed to be the primary cause of neuronal death. Accordingly, biochemical agents or surgery designed to reduce IOP are the current standard therapy. Nevertheless, lowering IOP is not always sufficient to stop neuronal loss. Moreover, optic nerve degeneration sometimes occurs in the absence of elevated IOP, a condition called normal tension glaucoma (occurring in approximately one third of glaucoma patients). Thus, neuroprotective therapy is considered appropriate. We used a model of chronic elevation in IOP of the rat to examine the ability of Cop 1 or PolyYE vaccination to attenuate the death of neurons which are under continuous stress conditions, as it might occur in ALS patients. Since

glaucoma is a chronic neurodegenerative disease as ALS, neuroprotection afforded in the glaucoma model may be indicative of a similar neuroprotection in ALS.

Induction of high IOP was performed as follows: Using a Haag-Streit slit lamp emitting blue-green argon laser irradiation, the right eye of anesthetized adult male

5 Lewis rats were treated by 80–120 applications directed towards three of the four episcleral veins and towards 270 degrees of the limbal plexus. The laser beam was applied with a power of 1 watt for 0.2 seconds, producing a spot size of 100 mm at the episcleral veins and 50 mm at the limbal plexus. At a second laser session one week later, the same parameters were used except that the spot size was 100 mm for

10 all applications. Irradiation was directed towards all four episcleral veins and 360 degrees of the limbal plexus 24.

To measure the elevation of IOP, the rats were injected intraperitoneally with 10 mg/ml acepromazine, a sedative drug that does not reduce IOP, and 5 minutes later the pressure in both eyes was measured using a Tono-Pen XL tonometer

15 (Automated Ophthalmics, Ellicott City, MD, USA), after applying Localin to the cornea. Average of 10 measurements taken from each eye was calculated. One week after the first laser treatment, the IOP reached levels of about 30 mmHg without any significant change until the end of the experiment (3 weeks after the first laser treatment) as shown in **Table 3** below.

20 To determine RGC survival, the hydrophilic neurotracer dye dextran tetramethylrhodamine (Rhodamine Dextran) (Molecular Probes, Oregon, USA) was applied 3 weeks after the first laser treatment directly into the intra-orbital portion of the optic nerve. Only axons that survive the high IOP and remain functional, and whose cell bodies are still alive, can take up the dye and demonstrate labeled RGCs.

25 The rats were killed 24 hours later and their retinas were excised, whole-mounted, and the labeled RGCs were counted under magnification of ×800 in a Zeiss fluorescent microscope. From each retina four fields were counted, all with the same diameter (0.076 mm^2) and at the same distance from the optic disk. RGCs were counted by an observer blinded to the identity of the retinas.

Table 3 summarizes the RGCs survival in rats with normal IOP and in rats with a laser-induced increase in IOP 3 weeks later.

Table 3

5

Normal	3 weeks post laser		
Mean RGCs ± SD (per mm ²)	Mean IOP± SD	Mean RGCs ± SD (per mm ²)	% Survival
2525 ± 372 (n = 5)	29.92 ± 2.38 (n = 10)	1420 ± 272	53.9

3a. Effect of PolyEY vaccination on RGC survival in the glaucoma IOP model

SPD rats were immunized with PolyEY (500 µg) emulsified with CFA one hour after the first laser treatment (n=9). One control group was immunized with 10 CFA without the antigen (n=7) and the second control group was injected with PBS alone (n=5). As shown in Fig. 3A, though the IOP remained elevated throughout the experimental period, PolyEY-, but not PBS-immunized rats, showed significant increased survival of their RGCs compared to non-immunized rats. Protection of RGC was calculated as percentage of cells survived in the treated groups out of the 15 total cell loss in the non-immunized group.

3b. Effect of Cop 1 vaccination on RGC survival in the glaucoma IOP model

Using the rat model of IOP-elevation, Cop 1 was shown to attenuate neuronal loss when given (500 µg in CFA) at the start of IOP elevation or a week later (see 20 Fig. 3B), despite the fact that the IOP remained high and nerve degeneration has already started. Additionally, Cop 1 vaccination, given together with the IOP-lowering drug brimonodine, resulted in greater RGC protection than using brimonodine alone (see Fig. 3B, insert).